The Protective Role of Tumour Necrosis Factor Alpha in the Heart

James J. Meiring

Submitted for the Degree of Master of Science in Medicine

Faculty of Medicine, University of Cape Town

2001

Supervisor Dr. M.N. Sack

Declaration

I, James Justus Meiring, hereby declare that the work on which this thesis is based is my original work (except where acknowledgement indicates otherwise), and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

I empower the University of Cape Town to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

_____

Signature

_____

Date

ACKNOWLEDGEMENTS

Acknowledgements

It is a pleasure to record my thanks to many individuals who provided assistance during this research.

A special thanks goes to Michael N. Sack, M.D. Ph.D. for supervision, encouragement and support. My sincere gratitude for the guidance that has enabled me to do this Masters after being a research assistant for five years. Thanks as well to Lionel H. Opie, MBBCh, Ph.D. D.Sc. for his scientific guidance and lectures. I also wish to thank D.L.L. Dietrich, Ph.D. for her scientific guidance and intellectual input.

In addition, I would like to sincerely thank my fellow researchers in the laboratory of M.N. Sack, both national and international, as well as the administrative and technical staff for their encouragement and technical support.

Furthermore I would like to express my thanks to WINETECH for the scholarship. They gave me financial security and therefore the peace of mind to achieve this Masters.

To my parents: Thank you for your love and support from start to finish. I dedicate this work to my father. Without you I would have never made it.
Also to my girlfriend: Thanks Karla.

Last, but not least, to my Heavenly Father: Thanks for your help.

TABLE OF CONTENTS

TABLE OF CONTENTS

LISTS OF FIGURES AND TABLES

LISTS OF FIGURES AND TABLES

pre-treatment (30 minutes exposure followed by 1 hour wash off) on lactate dehydrogenase (LDH) release in Girardi cells following an ischaemic insult (8 hours). Data is normalised to the ischaemic insult group (100%; n=6; *p<0.05), page 104.

Figure 10

Effect of NF-κB inhibition by salicylic acid (10 mM) and diethyldithiocarbamate (DDTC; 10 mM) on ischaemic preconditioning (cycles of 5 minutes global ischaemia followed by 5 minutes of reperfusion prior to the index regional ischaemia) and TNFα (0.5 ng/ml) pre-treatment (7 minutes followed by 10 minutes washout) on 30 minutes regional ischaemia (index ischaemia) followed by 2 hours reperfusion in the Langendorff perfused rat heart. Infarct size is expressed as a percentage of the area at risk, page 107.

PUBLICATIONS

PUBLICATIONS

MANUSCRIPT ACCEPTED FOR PUBLICATION:

2001 Relation of Cyclic Nucleotide Ratios to Ischaemic and Reperfusion Injury in Nitric Oxide Treated Rat Hearts. EF du Toit, ***James J Meiring***, Lionel H Opie.
Accepted for publication in the *Journal of Cardiovascular Pharmacology*

ARTICLES SUBMITTED FOR PUBLICATION:

2001 Ischaemic and Pharmacologic Preconditioning in Girardi Cells and in C2C12 Myotubes - Induce Mitochondrial Uncoupling Jan Minners, Lydia Lacerda, Joy McCarthy, ***James J Meiring***, Derek M Yellon, Lionel H Opie, Michael N Sack
Circulation Research, 2001; In press

CONFERENCES: POSTER / ORAL PRESENTATION

2000: TNFα Promotes Tolerance against Ischaemic Damage in Myocytes - Evaluation of the Putative Role of a NFκ B Activated Cell Survival Programme; Congress of the South African Heart Association, 27 November 2000, University of Stellenbosch, South Africa

2000: TNFα Promotes Tolerance against Ischaemic Damage in Myocytes - Evaluation of the Putative Role of a NFκ B Activated Cell Survival Programme; 3rd Annual Zeneca Research Day, 05 September 2000, University of Stellenbosch, South Africa

2000: TNFα Promotes Tolerance against Ischaemic Damage in Myocytes - Evaluation of the Putative Role of a NFκ B Activated Cell Survival Programme. 27th Annual Research Day, 05 October 2000, University of Cape Town, South Africa

1999: Role of the K_{ATP}-channel in the Cardioprotection by Nitric Oxide Donors, EF du Toit, ***J Meiring***, J McCarthy, LH Opie. The 8th International Congress on Cardiovascular Pharmacotherapy, Amsterdam; Netherlands – 28 March to 01 April 1999;

Abstract published (# 189) in *Cardiovascular Drugs and Therapy*: Vol 13 (1), March 1999

1998: The Relation of Tissue Cyclic Nucleotide Ratios to Ischaemic / Reperfusion Injury in Nitric Oxide Donor Treated Rat Hearts. The 25th Annual Research Day of the Department of Medicine October 1998, University of Cape Town, South Africa

1998: Relation of Tissue Cyclic Nucleotide Ratios to Ischaemic / Reperfusion Injury in Nitric Oxide Donor Treated Rat Hearts. EF Du Toit, ***J Meiring***, J McCarthy, LH Opie.

The 52nd Congress of the Physiology Society of South Africa, Rustenburg, September 1998

1998: Relation of Tissue Cyclic Nucleotide Ratios to Ischaemic / Reperfusion Injury in Nitric Oxide Donor Treated Rat Hearts. EF Du Toit, ***J Meiring***, J McCarthy, LH Opie.

The 16th World Congress of the International Society for Heart Research, Greece;

Abstract published (# 323) in the *Journal of Molecular and Cellular Cardiology*: May 1998, pA83

ABSTRACT

ABSTRACT

The pleiotropic cytokine tumour necrosis factor alpha (TNFα) is produced by the heart in response to the ischaemic preconditioning (PC) stimulus. We hypothesised that this endogenously produced peptide may play a role in activating the ischaemic PC mediated tolerance towards a subsequent ischaemic insult in muscle cells. To test this and to delineate the downstream signalling cascades mediating this programme we developed classic PC protocols in adherent mature murine C2C12 myotubes and in human cardiac derived Girardi cell lines. The C2C12 myotubes were preconditioned using either one hour of simulated ischaemia (SI) or the PC-mimetic adenosine (0.1 mM) or TNFα (0.5 ng/ml) followed by one hour of reoxygenation followed by an eight hour SI insult. Cell viability was assessed by measuring lactate dehydrogenase (LDH) release. Simulated ischaemia (SI), PC, adenosine and TNFα activated the PC programme and increased cell viability by 40±3%, 28±5% and 36±4% respectively compared to the SI controls ($p<0.005$ in all experiments, $n\geq4$ × 6 well plates in all groups). Cell viability was also evaluated by the measurement of propidium iodide uptake on flow cytometry. Preconditioning and TNFα enhanced cell viability with a reduction in propidium iodide uptake by 28% and 41% respectively versus the ischaemic controls. To evaluate whether TNFα activation of the nuclear regulatory protein nuclear factor kappa B (NFκ B) mediates this myocyte protection, the NFκ B antagonists diethyldithiocarbamate (DDTC 10mM) or sodium salicylate (SA 100μM) were co-administered with TNFα. The myocyte protective effect

of TNFα was significantly decrease with both antagonists, although not completely inhibited/blocked (DDTC – attenuated cell viability by 62±6% and SA by 45±5% compared to the TNFα preconditioned cells ($p<0.05$ vs SI controls and $p<0.05$ vs TNFα PC, with either antagonists). To confirm these data, TNFα was used as a PC-mimetic in the isolated Langendorff perfused rat heart (Langendorff) preparation. Infarct size was used as the end point. In parallel with cell culture studies, TNFα again conferred preconditioning induced cardioprotection with partial abrogation of these effects with the pharmacological antagonists of NFκ B. Thus, TNFα administration mimics the cytoprotective effects of ischaemic PC in cardiac, skeletal myocytes and in the isolated perfused rat heart. Moreover, these data support the role of TNFα production as an endogenous paracrine / autocrine signalling peptide which promotes myocyte cellular survival, in part, through activation of NFκ B.

ABBREVIATIONS

ABBREVIATIONS

ANOVA	- Analysis of Variance
AP-1	- Activator Protein A
ATP	- Adenosine Triphosphate
cDNA	- Complementary Deoxynucleic Acid
COX2	- Cyclooxygenase-2
DDTC	- Diethyldithiocarbamate
DNA	- Deoxynucleic Acid
EDTA	- Ethylenediaminetetra-Acetate
ERK	- Extracellular Regulated Kinase
FADD	- Fas-associated Protein with Death Domain
Gi	- Inhibitory G-Protein
GLUT4	- Insulin Sensitive Cardiac Enriched Glucose Transporter
HR	- Heart Rate
IL-1β	- Interleukin 1β
iNOS	- Inducible Nitric Oxide Synthase
IPC	- Ischaemic Preconditioning
IKK2	- I kappa Kinase
IKBα	- I kappa B- Inhibitor Protein
IKKα	- I Kappa B Kinase alpha Complex
IKKβ	- I kappa B Kinase beta Complex
JNK	- c-Jun NH_2 – Terminal Kinase
K_{ATP}	- Adenosine Triphosphate Sensitive Potassuim Channel
LDH	- Lactate Dehydrogenase
L-NA	- L-nitro Arginine
LPS	- Lipopolysaccharides
LVDP	- Left Ventricular Developing Pressure
MAPK	- Mitogen Activated Protein Kinase
mitoK_{ATP}	- Mitochondrial Adenosine Triphosphate Sensitive Potassuim Channel
Mn-SOD	- Manganese Superoxide Dismutase
Mn-SODmRNA	- Manganese Superoxide Dismutase Messenger Ribonucleic

Acid

MPG	- Mercaptopropionyl-glycine
NFκ B	- Nuclear Factor Kappa B
NO	- Nitric Oxide
PC	- Preconditioning
PKB	- Protein Kinase B
PKC	- Protein Kinase C
PTK	- Protein Tyrosine Kinase
ROS	- Reactive Oxygen Species
SA	- Sodium Salicylate
SAPK	- Stress Activated Protein Kinase
SI	- Simulated Ischaemia
SOD	- Superoxide Dismutase
SWOP	- Second Window of Preconditioning
TLR 4	- Tumour Necrosis Factor Like Receptor 4
TNFα	- Tumour Necrosis Factor-Alpha
TNFR1	- Tumour Necrosis Factor Receptor 1
TNFR2	- Tumour Necrosis Factor Receptor 2
TRAF2	-TNF Receptor Associated Factor 2
TTC	- 2,3,4,5-Triphenyl-Tetrazolium Chloride
5HD	- 5-Hydroxydecanoate

A. INTRODUCTION

A. INTRODUCTION

1. Preconditioning in the Heart

Most solid organs including the heart can be protected from ischaemic injury through pre-emptive programming called 'preconditioning'.[1] This phenomenon was first described by Murry, Jennings and Reimer at Duke University in 1986. At the time, it was assumed that repetitive short periods of myocardial ischaemia would have a cumulative deleterious effect on the heart resulting in progressive cell necrosis. Reimer and colleagues designed an experiment to explore the relative contribution of high energy phosphate depletion and catabolite accumulation on lethal cell injury in the canine myocardium. They found that repetitive brief episodes of regional ischaemia in anaesthetised dogs resulted in an attenuation of the reduction of adenosine triphosphate (ATP) levels during a subsequent prolonged ischaemic insult. This preservation of ATP was associated with a reduction in the size of the myocardial infarction compared to dogs not exposed to initial transient repetitive ischaemic insults. This phenomenon which they termed 'ischaemic preconditioning' was accompanied by ATP preservation and infarct size reduction.[1] It is known that the protection afforded by preconditioning ischaemia is apparent immediately following the preconditioning trigger but wanes after 1-2 hours. This is referred to as 'classical' preconditioning or the first window of protection. In 1993 Yellon and colleagues reported that after 12-24 hours following the preconditioning ischaemic 'trigger', a delayed phase of myocardial protection is induced which although not as powerful as the early phase, is more prolonged and lasts up to 72 hours.[2,3] This delayed

phase of resistance to ischaemic injury has been termed delayed preconditioning or the 'second window of protection' (SWOP). It is likely that these temporally distinct cyto-protected periods are mediated by different intracellular regulatory events even though they share the same preconditioning 'triggers'.[3-5] The current paradigm being that the 'classic' preconditioning programme is predominantly activated via post-translational modifications of peptides with associated sequelae in the cell. In contrast the delayed protection is proposed to be initiated at the pre-translational level. The classic preconditioning 'triggers' include transient coronary occlusion, brief periods of low-flow ischaemia, adenosine, opioids or bradykinin signalling, vigorous β-adrenergic stimulation or via cycles of calcium depletion and repletion.[6-9]

2. A Historical Perspective of Research on Preconditioning

2.1. The Energetic Hypothesis

An energetic hypotheses to explain PC was the original mechanism proposed and has recently been revisited. This hypothesis regarding ATP metabolism in preconditioning was based on the early studies of Murry , Jennings and Reimer in 1986.[1] These investigators showed that four periods of five minutes of coronary occlusion, each separated by five minutes of reperfusion, led to a reduction in infarct size following a subsequent 40 minutes occlusion from 30% of the area of risk in control animals to 7% in dogs exposed to the preconditioning protocol. The level of creatine phosphate was also reported to

be higher in preconditioned hearts during the first 10 minutes of ischaemia. Murry et al demonstrated that preconditioning resulted in less accumulation of glycolytic products, including lactate, during ischaemia. These data suggest that the preconditioning trigger results in a reduced rate of glycolysis and of ATP hydrolysis in the subsequent ischaemic insult. Studies also revealed attenuation in the development of intracellular acidosis following preconditioning.[10] Sequelae of this intracellular environment are postulated to lead to a reduction in ischaemia induced sodium and calcium loading on the basis of a limitation of sodium-proton exchange and sodium-calcium exchange during ischaemia following the PC 'trigger'. The paradigm suggesting a preservation of ATP and a reduction of glycolysis as the compensatory metabolic responses to the preconditioning trigger are probable incomplete. This was recently illustrated by Fryer et al, who demonstrated that ischaemic preconditioning (IPC) significantly increased the rate of ATP synthesis in rat hearts compared to non-preconditioned ischaemic hearts.[11] They also showed that 5-Hydroxydecanoate (5-HD), a putative mitochondrial ATP-sensitive potassium channel blocker partially attenuated the preservation of ATP synthesis when it was administered before IPC. Fryer et al's findings of improved rates of ATP synthesis in mitochondria isolated from preconditioned hearts are consistent with the mechanism that regulation of matrix volume is an essential element in the regulation of mitochondrial energy production, and matrix expansion secondary to mitochondrial K_{ATP} opening which has been postulated to activate electron transport and stimulate mitochondrial metabolism.[12,13] Furthermore glucose transport into cells is a tightly regulated process that responds to hormonal and other stimuli

to provide substrate for energy generation and for glycogen. Modulation of glucose transport could be important for the cardioprotective effects of brief intermittent periods of ischaemia and reperfusion or ischaemic preconditioning. Previously it was shown that preconditioning reduced the production of lactate with an attenuation of the fall in pH during the sustained ischaemia, suggesting that glucose utilisation is decreased during ischaemia in preconditioning.[10] However, in potential contrast to this hypothesis Tong *et al* reported that preconditioning stimulates glucose uptake and that this increased uptake was not blocked by wortmannin.[14] The translocation of GLUT 4 to the plasma membrane was also observed in rat hearts exposed to preconditioning. Moreover, Tong and colleagues showed that preconditioning stimulates phosphorylation of protein kinase B (PKB) and increases glucose uptake in a p38 MAP Kinase dependent manner. This increased glucose uptake would be postulated to support maintenance of ATP synthesis as reported above. In conclusion the metabolic perturbation in the preconditioning programme has not been fully clarified and requires additional work.

2.2. Cell Surface Ligands and G-Protein Coupled Receptors

Signal transduction pathways activated by the precondition 'trigger' have received greater scientific attention than the energetic hypothesis and a larger body of data has been generated in this arena. Here several lines of evidence suggest a role for a number of endogenous paracrine mediators, which are released during the brief period of ischaemia. These ligands act on local receptors, as triggers of both classical and delayed preconditioning. Various

models of preconditioning in different species have implicated the involvement of substances such as adenosine, acetylcholine, catecholamines, angiotensin II, bradykinin, endothelin and opioids.[3,15,16] The relative importance of these triggers seems to be dependent on the species and the end-points of protection studied.[9,17] A common signalling event downstream of these triggers is that they are coupled to a pertussis toxin sensitive inhibitory G-proteins (Gi) receptor. It has been proposed that activation of these Gi-protein coupled receptors by various ligands in turn results in activation of protein kinase C (PKC) which is thought to be an important intermediate regulatory kinase in the signal transduction pathway of ischaemic preconditioning.[9,16] In addition, Gi may mediate other potentially protective mechanisms, such as direct inhibition of L-calcium channels and activation of ATP-sensitive potassium channels.

2.3. Activation of Kinases

Downey and colleagues have proposed that protein kinase C (PKC) plays a major role in the signal transduction of preconditioning. PKC is thought to play an important role in transmitting the effects of several agonists linked to phospholipase C and the phosphatidyl inositol systems.[18-20] PKC has several isoforms of which a few may be crucial in preconditioning.[21] It is believed that adenosine A1 receptor stimulation activates the δ-protein kinase C isoform. Some studies have supported the hypothesis originally proposed by Downey that IPC could be blocked by pretreatment with specific PKC inhibitors and the substitution of IPC with PKC activators mimics the infarct limiting effects of IPC. The time course of classical preconditioning also

parallels the transient translocation of PKC to the sarcolemmal membrane following activation.[22] However, the putative peptide end-effector(s) of preconditioning which are phosphorylated during the activation of the PKC has yet to be determined. It is also likely that other protein kinases, in parallel with or following activation by PKC may play a role in classic PC.[23] Another downstream target of PKC-dependent signalling during the development of PC is the Mitogen Activated Protein Kinase (MAPK) superfamily, which includes 3 major subfamilies: the p44/p42 MAPKs (Extracellular Regulated Kinases, ERKs), the p38 MAPKs and the p46/p54 MAPKs (Janus Kinase Janus Kinases, JNKs).[18,24,25] Studies have shown that PC activates all of the 3 MAPKs subfamilies. Chelerythrine abolished the activation of p44/p42 MAPKs that indicated that MAPK are downstream of PKC. Overexpression of certain PKC isoforms also leads to the activation of p44/p42 MAPKs.[25]

2.4. Transcription Factors

The recruitment by the PC stimulus of PKC and certainly other as-yet-unidentified kinases are thought to lead to the activation of transcription factors that govern the expression of the cardioprotective genes responsible for delayed PC. The first transcription-regulator element to be identified as an integral component of PC response was nuclear factor-κB (NFκ B),[4] which is known to transactivate for e.g. Inducible Nitric Oxide Synthase (iNOS), COX-2 and aldose reductase gene expression.[3,26,27] Xuan et al demonstrated that ischaemic PC induces rapid activation of NFκ B and that this event can be mimicked by infusing NO donors in the absence of ischaemia.[4] Inhibition of

NFκB with diethyldithiocarbamate completely abrogated the cardioprotective effects of PC indicating that NFκB plays a critical role in the genesis of delayed PC. The ischaemic PC-induced activation of NFκB was blocked by treatment with reactive oxygen species agonist, L-NA, MPG, and LD-A, indicating that the cellular mechanism whereby ischaemic PC activates NFκB involves the formation of NO[28-30] and Reactive Oxygen Species (ROS)[31] and the subsequent activation of Protein Kinase C (PKC) - and Protein Tyrosine Kinase (PTK) -dependent signalling events.[22,32,33] Subsequent studies have shown that ischaemic PC induces both serine and tyrosine phosphorylation of IκBα (the cytosolic chaperone of NFκB) concomitant with PKC-dependent activation of IKKα and IKKβ, suggesting that a dual mechanism accounts for the activation of NFκB during ischaemic PC.[3] In addition, activating protein 1 (AP-1) has been found to be activated by brief myocardial ischaemia in rats and by overexpression of PKCε in cardiomyocytes.[34] Whether this transcription factor plays a functional role in the development of PC response remains to be elucidated. It seems likely that the upregulation of iNOS, COX-2 and other co-mediators after PC stimulus involves simultaneous activation of multiple stress-responsive transcription factors acting in an additive or synergistic manner.

2.5. K_{ATP} Channels – A Common Denominator/Mediator of Preconditioning

A number of ligands and signalling pathways have been proposed to be involved in mediating the cardioprotective effect of IPC, as described above.

Moreover, evidence suggests that the ATP-sensitive potassium channel (K_{ATP} channel) is an important component of this phenomenon and may serve as a mitochondrial end effector/mediator in this process.[6,13,35-37] A number of K_{ATP} channel openers have been shown to produce a beneficial effect on the myocardium in numerous models of ischaemia and K_{ATP} channel activation has been demonstrated to be a key component of IPC. Authors have shown that two K_{ATP} channel antagonists, glibenclamide and sodium 5-HD blocked the protection produced by IPC and also demonstrated that the K_{ATP} channel openers, aprikalim, diazoxide and nicorandil mimick the beneficial effect of IPC by reducing infarct size. Infarct size is considered the gold standard in defining the phenomenon of IPC. There is however a lot of speculation concerning the role of the sarcolemmal versus mitochondrial K_{ATP} channels in conferring this cardioprotection.[6] K_{ATP} channel openers that work on both for example pinacidil, cromakalim and P-1075 have mimicked the cardioprotection of IPC. It is hypothesised that opening of the mitochondrial K_{ATP} channel can result in the following phenotypic consequences: membrane depolarisation, matrix swelling, enhanced respiration and reduced calcium overload.[12] In addition, numerous investigators believe that activation of the mitochondrial K_{ATP} channel results in ROS production, which act as intracellular signalling intermediates to modulate downstream cardioprotective events.[38,39] Despite all of the studies to date, the mechanistic consequences of mitochondrial K_{ATP} channel activation in the IPC programme require additional investigation.[40]

2.6. Alternative Strategies

The majority of investigation concerning preconditioning has focused on the study of G-protein receptor coupled signalling pathways.[9] However the biology of the cytoprotective programme directing preconditioning remains elusive. A question arising is whether G-protein independent receptor mediated pathways could play a role in orchestrating the preconditioning programme. Furthermore, investigation of alternative signalling pathways could identify novel events in the cytoprotective programme. In this regard a parallel innate biological phenomenon that could promote cytoprotection against ischaemia has recently been recognised.[41,42] The process has been postulated to be a component of the primative innate immune system.

3. Innate Immunity - A Potential Component of the Preconditioning Programme?

The innate or natural (non-specific) immune system is the primitive activation of cellular events to protect the host against adverse events, including the invasion of pathogens. These adaptive mechanisms include the production of heat (fever) and the activation of an the inflammatory response which involves the action of several molecules including histamines, kinins, cytokines, chemokines, neutrophils and mast cells among others to inactivate/destroy pathogens.[43] These innate responses not only destroy pathogens, but also prime cells to resist subsequent attack and regulate additional components of

the immune response. To activate innate immunity phylogenetically conserved receptors, termed pattern-recognition receptors that have the ability to recognise specific pathogen-associated molecular patterns are evident in myeloid and non-myeloid cells.[44] Examples of the pathogen associated molecular pattern include the lipopolysaccharides of Gram-negative organisms and the teichoic acids of Gram-positive organisms.[45] One of the more interesting examples of a pattern-recognition receptor is the recently described Tol/nuclear factor (NF)-κB host defence pathway that was first described in the fruit-fly *Drosophila*.[46] There is substantial evidence demonstrating mediators and effectors of the innate immune response, including proinflammatory cytokines, nitric oxide and chemokines can be endogenously produced in the adult mammalian heart (cardiomyocytes) in response to challenges with classical pathogen-associated molecular patterns, such as lipopolysaccharides and viral particles.[42] Moreover, it has recently been demonstrated that the heart also expresses two pattern-recognition receptors for pathogen-associated molecular patterns namely CD14 and TLR-4.[44] Recent observations suggest that TLR-4 is critical for lipopolysaccharide-mediated activation of proinflammatory cytokine expression in the adult mammalian heart. These observations provide presumptive evidence for a functionally intact innate immune system in the heart. Medzhitov et al were the first to show that constitutively active TLR-4 induces the activation of NFκ B.[47] TLR-4 can also induce c-Jun NH_2 – terminal kinase (JNK). TLR's may serve as pro-inflammatory receptors in cell types without a dedicated immune function. Injured human and murine myocardium exhibit focal areas of intense TLR4 expression. Microorganisms

are not the only activators of the innate immunity receptors, but also endogenous signals that originate from injured cells that emanate "danger signals" can activate this immune response. Oxidative stress during ischaemia is known to induce cell death by apoptotic and necrotic pathways and to trigger pro-inflammatory signalling pathways that activate NFκ B and AP-1 transcription factors.[48] Hypothetically, then the ischaemia in the preconditioning 'trigger' may activate the innate immune programme. To evaluate this, we reviewed the literature to establish whether known activators of innate immunity have been demonstrated to activate the preconditioning phenotype. One activator of innate immunity (i.e. endotoxin)[49] and one apical factor directing innate immunity (i.e. TNFα) has been shown to mimic delayed preconditioning.[50] These effects are discussed below.

4. Endotoxins in the Second Window of Protection (SWOP)

A phenomenon called endotoxin tolerance has been described where a subset of endotoxin driven responses are down regulated after an initial exposure to endotoxin.[51] This phenomenon is proposed to provide protection from the uncontrolled immunological activation during acute endotoxic shock. This endotoxin tolerance syndrome shares a remarkably similar pattern to that of ischaemic preconditioning. This similarity is supported by the fact that endotoxin tolerance has been shown to promote tolerance to brain ischaemia, with attenuated thermal responses and reduced mortality in some species.[52] Similarly pretreatment of rats with endotoxin 24 hours before regional ischaemia results in a reduction in infarct size similar to that found with

delayed preconditioning. Endotoxin pretreatment in vivo increases the mitochondrial respiratory capacity in rat hepatocytes. Mitochondrial biogenesis and the subsequent increase in both enzymatic scavenging of superoxide anion is a central feature of endotoxin-mediated tolerance to oxidative stress.[53] Delayed preconditioning involved the production of protective proteins including Manganese superoxide dismutase (Mn-SOD).[3] Further knowledge on tolerance mechanisms is necessary in order to achieve a comprehensive view of this phenomenon. Cardioprotection by ischaemic preconditioning and by lipopolysaccharides is associated with a reduction in TNFα, following the ischaemia/reperfusion injury.[49] It is believed that lipopolysaccharides (LPS) induced ecto-5'-nucleotidase activity and subsequently results in increased production of adenosine which could then provide cardioprotection. In addition to causing a time-dependent induction of TNFα, LPS also induce IL-1β and MnSOD mRNA content in rat heart. LPS also causes upregulation of the expression of intercellular adhesion molecule-1 and P-selectin. Endotoxins can activate macrophages, monocytes and cardiac myocytes, which then display enhanced cytotoxic activity and synthesis of proinflammatory cytokines including TNFα. As TNFα is pleiotropic with diverse effects that include cytoprotection and apoptosis, a question that could arise is whether this cytokine can trigger the preconditioning cytoprotective programme?

5. TNFα in Second Window of Preconditioning

In order to answer the above question, investigators have studied whether TNFα can trigger the second window of protection (SWOP). The second window of ischaemic preconditioning is closely related to *de novo* synthesis of proteins such as heat shock proteins, and the endogenous free radical scavenger mitochondrial manganese superoxide dismutase (Mn-SOD). Interestingly TNFα and interleukin 1 (IL-1) are known as potent inducers of Mn-SOD mRNA.[54] TNFα and IL-1 are both reported to be involved in the radioresistance cytoprotection induced by sublethal ionizing radiation or lipopolysaccharide. These cytokines are also reported to be involved in the delayed cardioprotection against ischaemia / reperfusion injury in rat and canine models. Yamashita reported that TNFα and IL-1 induce a biphasic cardioprotection, which corresponds to the activation of Mn-SOD mRNA. In a separate study Yamashita and colleagues demonstrated that the induction of TNFα and IL-1β during exercise plays an important role in the exercise – induced cardioprotection through the activation and or the induction of MN-SOD. In a subsequent study Yamashita and colleagues demonstrated that they could abrogate delayed ischaemic preconditioning in vivo by administration of antisense to Mn-SOD and via pretreatment with neutralising antibodies to TNFα and IL-1β. They concluded that both TNFα and IL-1β are involved in the ischaemia-induced delayed cardioprotection via the induction and activation of Mn-SOD. The signalling pathway of TNFα and IL-1β trans-activation of Mn-SOD was recently shown to occur via activation of NFκ-B.

6. TNFα and Classic Preconditioning

Data regarding TNFα in classic PC is limited. In brief, Meldrum et al demonstrated that ischaemic preconditioning or adenosine pretreatment reduces TNF peptide in the myocardium following a subsequent ischaemia-reperfusion injury.[55] Heusch and colleagues demonstrated that serum TNFα levels are elevated in response to IPC trigger and then remained unchanged in rabbits subjected to IP or LPS compared to the control group where there was a marked increase during the ischaemia-reperfusion.[49] Another study where the role of TNFα in preconditioning was studied was when Hallenback and colleagues investigated the role of TNFα in hypoxic preconditioning.[56] They found that hypoxic preconditioning was attenuated by a TNFα neutralising antibody suggesting a possible protective role for TNFα in this signalling.

7. TNFα Signalling

Tumour necrosis factor-alpha (TNFα) is an apical cytokine with a multitude of functions and has been implicated in the pathophysiology of various diseases. It is known that TNFα mediates processes like inflammation, cellular survival, growth, differentiation and apoptosis (Reviewed[57,58]). TNFα belongs to a family of signalling molecules that exist as type II membrane proteins characterised by the C-terminus being extra-cytoplasmic. TNFα receptors signal as homotrimers and can exist either as membrane-bound or as truncated soluble forms. Two distinct surface receptors mediate the effects of

TNFα namely TNF receptor 1 (TNFR1 or p55) and TNF receptor 2 (TNFR2). TNFR 1 is the main receptor subtype in most cells types including the heart and its complex and divergent downstream signalling systems have been extensively studied. The apoptotic signalling events subsequent to receptor clustering have been well defined for Fas and TNFR1. The coupling of the adapter Fas-associated death domain protein (FADD) to TNFR1 is associated with the recruitment and activation of apoptotic proteases with subsequent progression to programmed cell death. The transduction of signals from TNFR2 and its role in TNFα signalling remains less well characterised. TNFR2 is believed to activate TNF receptor associated factor 2 (TRAF2) that also couples to TNFR1. TRAF2 is believed to be a cytoprotective complex that activates both NFκ B-dependent and – independent transcriptional events implicated in the induction of cytoprotective genes. These cytoprotective genes are involved in cellular growth, survival and proliferation. The cytoprotective pathways downstream of the TNFα receptors that may be relevant to the maintenance of cardiac homeostasis are protein kinases (PKC); stress activated protein kinases (SAPK) and downstream of c-Jun N-terminal kinase (JNK). The putative TNFα mediated cardioprotective programmes against ischaemia-reperfusion injury may be downstream of PKC, NFκ B and SAPK. Myocardial TNFα production has been well documented during acute ischaemia with or without reperfusion.[59,60] It is believed that the acute TNFα induced reduction in contractility during acute ischaemic syndromes may be an additional adaptive action of this pleiotropic peptide. Mann and colleagues demonstrated the cardioprotective action of TNFα in an acute ischaemia model using receptor knockout mice.[41] These

mice developed larger myocardial infarcts compared to wild-type littermate controls when subjected to an acute infarction in vivo. This study demonstrated that TNFα signalling is required for endogenous myocardial resistance to ischaemic cell death through an unknown mechanism. In our laboratory we have demonstrated using genetically modified mice that the TNFα signalling cascade plays a role in the postnatal adaptive myocardial growth response to multiple biomechanical stresses.[61] Moreover, pretreatment of rabbits with intravenous TNFα 24 hours before ex vivo simulated ischaemia and reperfusion resulted in improved cardiac contractile functional recovery and reduced lactate dehydrogenase release.[50] In our laboratory, Lecour et al have reported that pretreating isolated rat hearts with TNFα (0.5 ng/ml) for 7 minutes followed by a washout of 10 minutes prior to a simulated ischaemic-reperfusion protocol resulted in a marked increase in recovery with the TNFα group compared to the vehicle pretreated control.[62] Collectively, these data suggest that the production of TNFα by the myocardium in response to an ischaemic and reperfusion insult may in fact be an endogenous pathway activated by the heart to induce short-term intrinsic cardioprotection against subsequent ischaemia-reperfusion injury. Supportive evidence in brain tissue demonstrates that TNFα plays a key role in ischaemic tolerance.[56] The signalling mechanisms whereby TNFα may provide tolerance to ischaemia or to promote adaptive cardiac protection have, however, not been established.

8. Hypothesis

Thus for this dissertation, we hypothesise that TNFα is an endogenous trigger of classic preconditioning. Moreover, we believe that investigation of TNFα signalling may identify novel mechanism to enhance protection against ischaemic injury. To investigate the hypothesis we will undertake the following studies.

9. Objectives

- Delineation of the temporal and dose requirement of TNFα to promote protection against ischaemic injury;

- Evaluate whether this PC effect is via classic IPC or via novel signalling pathways.

B. METHODOLOGY

B. METHODOLOGY

1. Establishment of cell lines to study TNFα as a ligand to induce preconditioning

In order to evaluate our hypothesis and to interrogate the putative signalling cascade activated by TNFα in the heart, we began by using a cellular model, which simulated ischaemia and ischaemic preconditioning. We decided to perform our initial dose and temporal effects studies of TNFα in two cell lines that had previously been used for preconditioning studies i.e. mouse skeletal muscle cell line (C2C12)[63] and a human cardiac derived cell line, Girardi cells (Hela characteristics).[64] The C2C12 mouse cell line was subcloned from skeletal myotubes and was established by Yaffe and Saxel using hindlimb muscle.[65,66] The C2C12 cell lines are rapid dividing myoblastic cells that with differentiation develop multinucleated myotubes and characteristically produce muscle peptides. The second cell line used was the Human derived Girardi cell line. These transformed cells were derived from a biopsy specimen of right auricular appendage of an adult human heart. The cell was found to have Hela profile after DNA fingerprinting. These cells have epithelial cell morphology and grow as adherent monolayers.

2. Culturing of Cells

Both cell lines were obtained from the European Collection of Cell Cultures (Centre for Applied Microbiology and Research, UK) and stored in Liquid N_2.

To grow the cells, the cryovials were half immersed in 37-degree water bath for 30-40 seconds. The content of the ampule was pipetted out into a $25cm^3$ flask containing fresh culture medium. The cells were grown in these flasks until confluency was reached. The trypsin/EDTA solution contained 0.25% (w/v) Trypsin, 0.2% (w/v) EDTA and made up to volume with sterile phosphate buffer saline. The EDTA (disodium ethylenediamine tetraacetic acid) is a chelating agent to prevent the trypsin from binding to calcium and magnesium ions. The adherent cells were displaced by trypsin treatment and were then reseeded into secondary cultures. A cell passage constitutes the splitting of cells with trypsin following growth to confluence. When confluent, the cells were trypsinised with a trypsin / EDTA solution for 1-2 minutes in 37 ^0C, 5% CO2 incubator. Sufficient trypsin was added to ensure the whole layer of cells was covered. Cell detachment was confirmed using light microscopy (Inverted microscopy). Following detachment, serum or medium containing serum was added to inhibit the trypsin activity. Excess exposure of cells to trypsin is deleterious and trysinisation should be limited to a maximum of 1-2 minutes. The cells were then spun down in a centrifuge for 5 minutes at 1000 rpm. The trypsin is then aspirated off and the cell pellet is resuspended in sufficient volume of media to split into either 1:3 or 1:4 depending on the size of flask/plates used. Cell numbers are determined by cell counting on a haemocytometer. The number of cells per millimeter = the average count of cells per square multiplied by the dilution factor multiplied by the factor 10^4. The cells were plated to reach confluency in 24 to 36 hours.

3. C2C12 Cells

The C2C12 myotubes are maintained in medium that consist of Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1,5 g/L sodium bicarbonate, 4,5 g/L glucose and 1,0 mM sodium pyruvate and 10 % horse serum. The minimum seeding density for C2C12 cells is 20 $\times 10^3$ cells/cm^2. The doubling time for C2C12 is roughly 26 hours. Approximately 40 $\times 10^3$ cells/cm^2 constitute confluency. The C2C12 myotubes are split at a confluency of between 70-80% to prevent constitutive differentiation. To differentiate the cells into myotubes, the myoblasts media is changed to 2% horse serum when confluency reaches eg approximately 70-80%. The 2% horse serum replaces the foetal bovine serum. The C2C12 are only used 8-10 days post differentiation. During the differentiation of the C2C12 myoblast, the cells are refed every three to four days.

4. Girardi cells

Girardi cell media consists of Minimal Essential Medium, 10% fetal bovine serum and 2 mM L-glutamine. The Girardi cells are grown to 100% confluency for experimental purposes or prior to splitting. The minimum seeding density is 20×10^3 cells/cm^2. The doubling time for Girardi cells is 24 hours. When confluent the cell density is 160×10^3 cells/cm^2. They are grown in Minimal Essential medium with 10% Fetal Bovine serum. After reaching confluency they are split for plating in flask or in plates for experimental purpose.

5. Cell Viability Assays

5.1 LDH assay

To study the role of TNFα in protection we used lactate dehydrogenase (LDH) enzyme leakage into the media as one of the measurements of cell viability in all cell culture experiments.[21,67] LDH measurements in the media were measured using the Roche Molecular Diagnostics kit (LDH SFBC cat. 0736570). A control serum N (human) and control serum P was run concurrently with the experimental samples. Samples were run on the Cobas Mira spectrophotometer from Roche Diagnostics. Lactate dehydrogenase is the enzyme responsible for the conversion of lactate to pyruvate during aerobic conditions, and the reverse reaction during hypoxia. There are five LDH isoenzymes, and the rate of release of these enzymes into the blood stream is an indication of the degree of myocardial injury.[68] The samples are spectrophotometrically measured on the Cobas Mira at a wavelength of 340 nm.

5.2 Flow Cytometric Assessment of Propidium Iodide exclusion

We also used propidium iodide exclusion as an index of cell viability.[64,67] Propidium iodide (PI) is known to bind to DNA and become fluorescent. However, PI cannot enter cells with intact cell membranes, and is hence included in immunofluorescent staining protocols to identify dead cells. The acronym FACS (Fluorescene Activated Cell Sorting) and flow cytometry are used interchangeably. It works on the basis that individual cells held in a thin stream of fluid are passed through one or more laser beams causing light to scatter and fluorescent dyes to emit light at variuos frequencies.

Photomultiplier tubes (PMT) convert light to electrical signals and cell data is collected. At the end of the 8 hour ischaemic period, medium from each well was aspirated and saved. Cells were washed twice with warm PBS and then incubated for 2 minutes with 0.25% trypsin in 1mM EDTA. Detached cells were resuspended in the saved medium and then centrifuged at 3000rpm for 4 minutes in a pre-cooled benchtop centrifuge at 4^0C. The resulting cell pellet was then resuspended in ice-cold PBS, containing 1μg/ml propidium iodide. The cell suspension was then immediately run on a multichannel flow cytometer. The propidium iodide fluorescence was measured in a population of 1×10^4 cells and was expressed as percent change over ischaemic controls.

6. Establishment of Protocol to Mimic Ischaemia

The first objective was to establish a simulated ischaemia protocol in both cell lines. The normoxic and hypoxic buffer were based on buffers devised by Esumi *et al.*[69] The normoxic buffer consists of 137 mM NaCL, 3.58 mM KCl, 0.49 MgCl, 0.9 mM CaCl2, 4 mM Hepes, 5.6 mM glucose and pH of 7.4. The hypoxic buffer was as follows: 137 mM NaCL, 3.58 mM KCl, 0.49 MgCl, 0.9 mM CaCl2, 4 mM Hepes, and 20 mM 2-DG. The pH of the hypoxic buffer was adjusted to pH 6.4.

7. Simulated Ischaemia in C2C12 Cells

Marban and colleagues found that the C2C12 myoblast could not be protected by preconditioning, however it was possible to precondition myotubes.[63] Hence we used mature myotubes and the simulated ischaemia protocol was modified until the desired ischaemic insult could be obtained.

The optimal simulated ischaemia was obtained using the following protocol (Fig.1.1.): The control group was incubated in normoxic media throughout and maintained in a 5% CO_2 incubator. The simulated ischaemia group had normoxic media for the first 2 hours followed by 8 hours of ischaemic buffer. During simulated ischaemia the plates were maintained in a hypoxic incubator (5% CO_2, <1% O, 95% N_2). The IPC group was initially exposed to 1 hour of SI and 1 hour of normoxia and a subsequent 8 hours in the hypoxic incubator. Cell viability was assessed using LDH release into the medium of the experiment in all the groups. The identical temporal conditions for stimulated ischaemia were employed when using the Girardi cells. Cell viability was assessed by LDH and propidium iodide exclusion assay.

8. Protocol for IPC in C2C12 and Girardi Cells

To induce IPC in the C2C12 cells, the cells were maintained under hypoxic conditions for 1 hour followed by an hour of exposure to normoxic media prior to the 8 hours simulated ischaemia period (see *Fig 1.1*). To induce IPC in the Girardi cells, the cells were maintained under hypoxic conditions for 30 minutes followed by a 1 hour normoxia period prior to the 8 hours simulated ischaemia period (see *Fig. 1.2*).

Figure 1.1

Schematic representation of the protocols used in the C2C12 myotube study. All measurements of lactate dehydrogenase activity in the media were made at the end of the protocols as represented by the black arrows.

Fig. 1.1

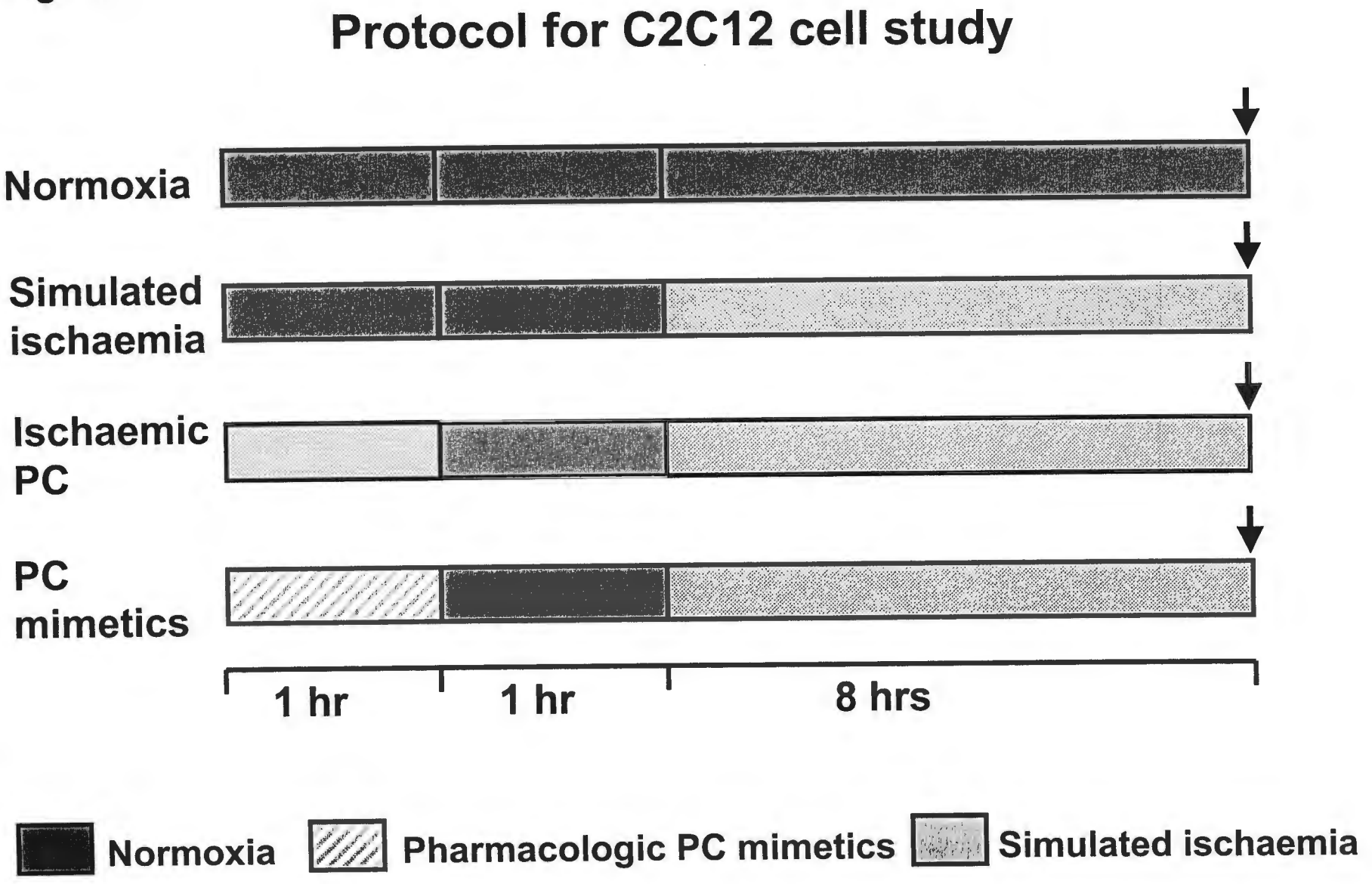
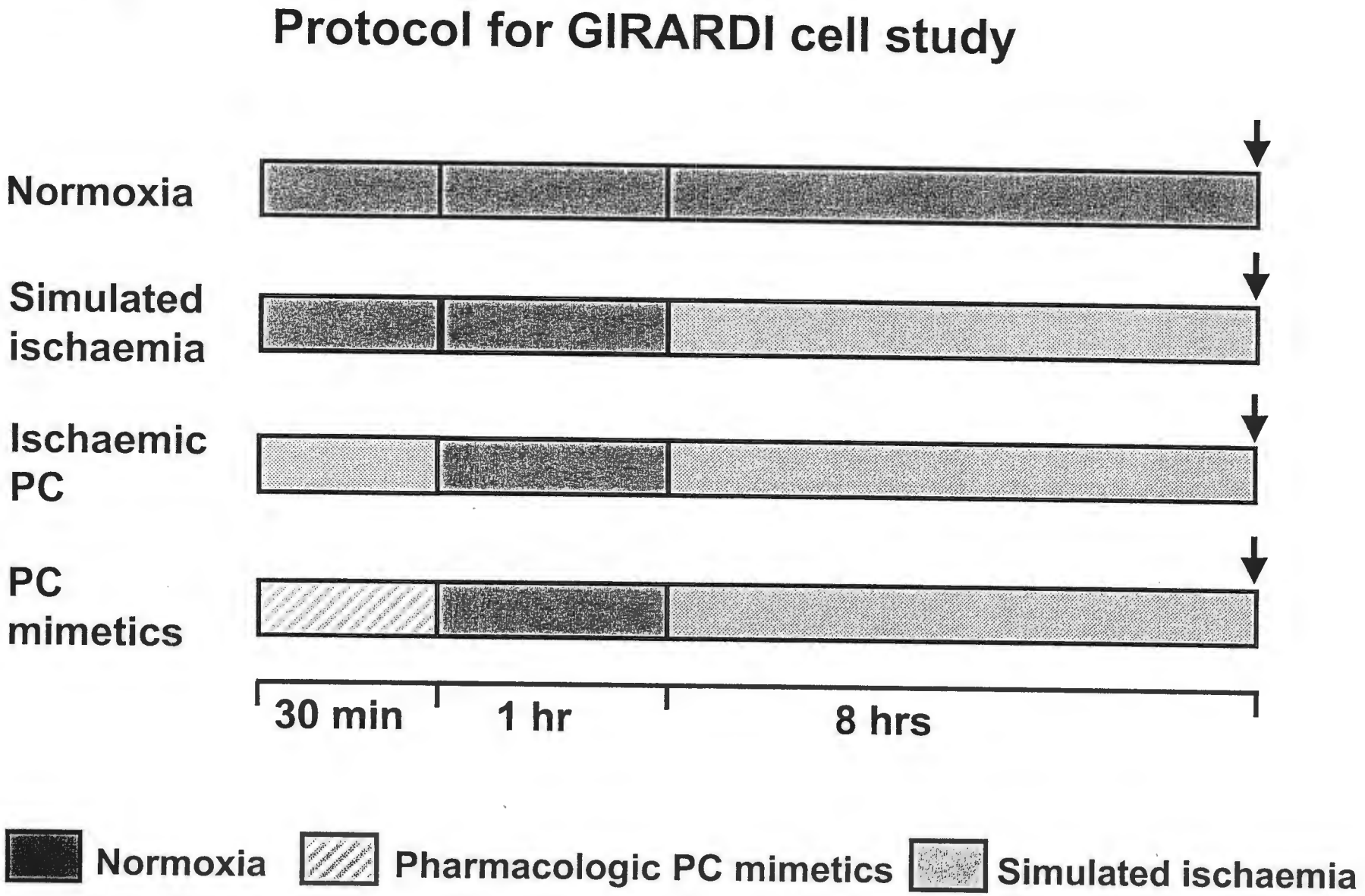

Protocol for C2C12 cell study
Normoxia
Simulated ischaemia
Ischaemic PC
PC mimetics
1 hr
1 hr
8 hrs
Normoxia
Pharmacologic PC mimetics
Simulated ischaemia

Figure 1.2

Schematic representation of the protocols used in the Girardi cell study. All measurements of lactate dehydrogenase activity in the media were made at the end of the protocols as represented by the black arrows. To confirm cell viability the PI exclusion assay was used and assess with FACS Analysis following the 8 hour ischaemic period.

Fig. 1.2


Protocol for GIRARDI cell study
Normoxia
Simulated ischaemia
Ischaemic PC
PC mimetics
30 min
1 hr
8 hrs
Normoxia
Pharmacologic PC mimetics
Simulated ischaemia

9. Dose and Temporal Effect of TNFα in Promoting Cytoprotection

Various TNFα concentrations were tested to evaluate the temporal requirements and dose effect to confer protection against ischaemic injury. The dose range was initially established from a literature search, which demonstrated the spectrum of survival or deleterious effects of TNFα. A dose response curve was set up that ranged from as low as 0.005 to 20 ng/ml TNFα. The cells were also incubated with the TNFα at various intervals. TNFα was incubated with cells as a PC mimetic, as a pretreatment and during hypoxia. After the ideal TNFα concentration was established, the cells were incubated with the desired dose as a pretreatment agent. It is the period prior to the eight hours hypoxia period. The cells were also incubated during the eight hours hypoxia with TNFα. The NFκ B blockers were used along with the TNFα during the PC mimetic as well as during the pretreatment phase and the 8 hours hypoxic phase. Known pharmacological blockers of the IPC programme such as chelerythrine (PKC inhibitor), 5HD (mitochondrial K_{ATP} channel blocker) and glibenclamide (sarcolemmal and mitochondrial K_{ATP} channel blocker) were incubated together with TNFα or during the IPC protocol.

10. Isolated Langendorff Perfused Rat Heart Model

Once we established the optimal dose of TNFα as an IPC mimetic we then began to evaluate the signalling cascade downstream of TNFα in the isolated perfused rat heart preparation. We switched to the isolated heart due to its greater physiological relevance and due to the fact that it is ex-vivo versus the

in-vitro cell system. The isolated perfused heart apparatus used was first described by Langendorff (1895), and later modified by Neely and co-workers, (1967).[70]

Animal used: Male Long-Evans rats weighing 270-380 grams and fed a standard rat chow diet were used for all experiments performed in this study. All animals received care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by National Institutes of Health (NIM Publication No. 80-23, revised 1996). Rats were intraperitoneally anaesthetised with 0.35ml sodium pentobarbitone and then intravenously heparinised (200 IU) through the exposed femoral artery. The hearts were then rapidly excised, immersed in 4^0 C Krebs-Henselet buffer for transfer to the perfusion apparatus. Arrest was induced to afford the hearts maximal protection during the brief ischaemic episode during the transfer to the perfusion apparatus. The aorta was cannulated and perfusion re-established within 1 minute of excision from the donor animal. Hearts were retrogradely perfused at a constant pressure (100 cm H_2O) and temperature (37^0C) with a modified Krebs-Henseleit buffer containing (mM) NaCl 118.5, $NaHCO_3$ 25, KCl 4.75, $MgSO_4$ 1.19, KH_2PO_4 1.18, CaCl2 1.36 and glucose 11. Oxygenation of the perfusion medium with 95% O_2 and 5% CO_2 resulted in PCO_2 of approximately 40 mmHg, PO_2 of approximately 550 mmHg and pH7.4. The pulmonary artery was incised to allow free drainage of the coronary effluent. Hearts were maintained at 37^0 C throughout the experiment

by surrounding them with a water jacketed chamber maintained at this temperature. A compliant balloon was inserted into the left ventricle to measure left ventricular end diastolic pressure, systolic pressure, heart rate and coronary flow. These functional parameters were recorded throughout the experiment using a pressure transducer which was connected to a Lectromed chart recorder or a computerised bridge amplifier / digitiser (PowerLab / 400, AD Instruments, Sydney, Australia), and continuously recorded using a Windows 95 operating system and PowerLab software. The criteria used for the inclusion of perfused rat hearts was a heart rate between 240-410 beats/min, systolic pressure between 70-120 mmHg, end diastolic pressure of 4-10 mmHg and a rate pressure product between 21 000-36 000 (mmHg beats/min)$\times 10^3$. In addition, only hearts with a coronary flow 8-18 ml/min were used.[71]

11. Coronary Occlusion

A 4/0 silk sature was placed around the left coronary artery, close to its origin and the hearts were threaded through the tip of a Gilson pipette to produce a snare and locked with a second pipette tip. Regional ischaemia was induced by carefully tightening the silk suture of the pipette against the epicardial surface; 30 minutes of regional ischaemia was followed by 120 minutes of reperfusion. Heart rate (HR) and left ventricular developed pressure (LVDP=difference between LV systolic and diastolic pressures) were continuously documented on the Lectromed recorder. Coronary flow was measured at regular intervals throughout the experiment.

12. The Perfusion Protocol

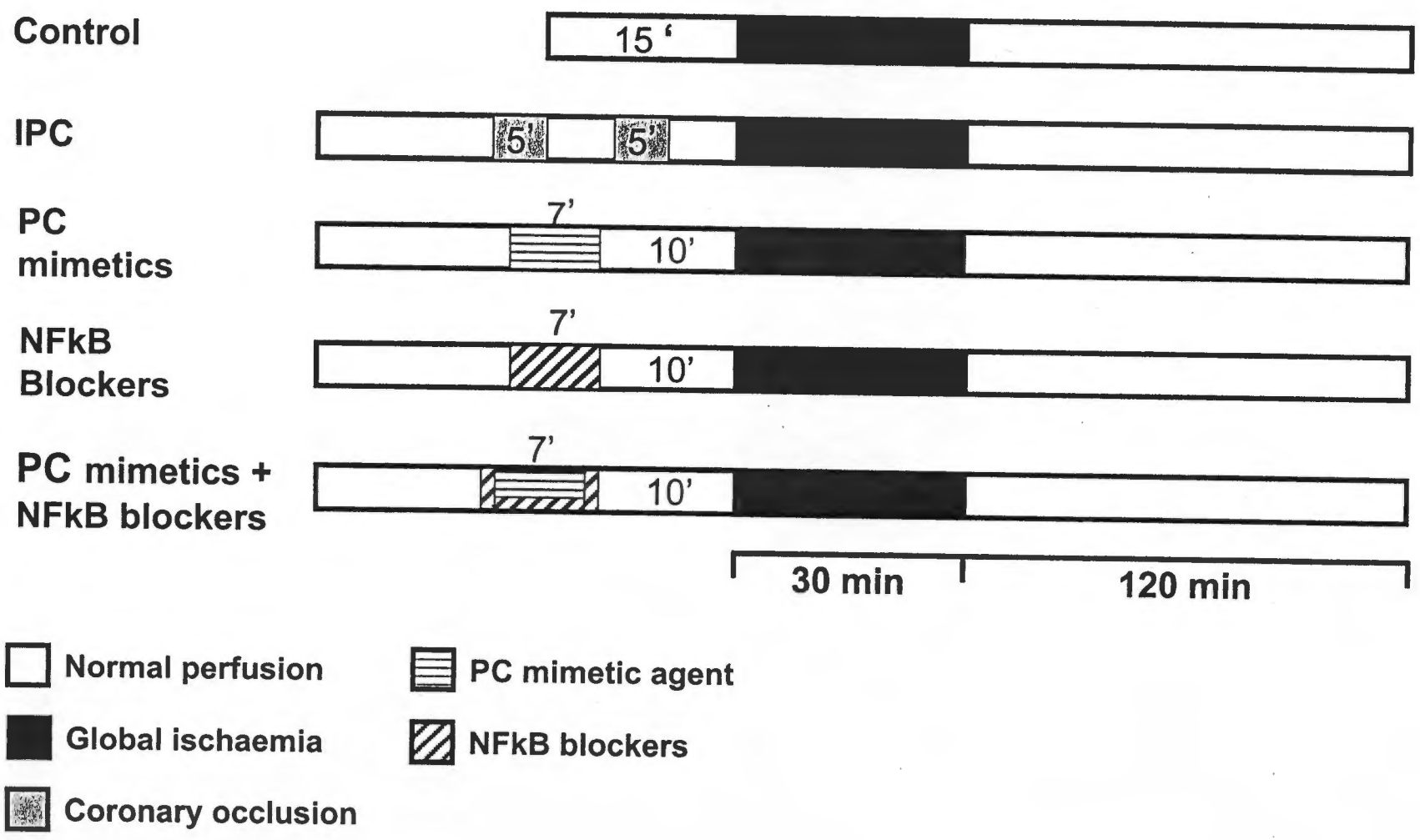
The perfusion protocol is shown in *Fig.* 2. All hearts were allowed an equilibration period of at least 15 minutes and were consequently subjected to 30 minutes of regional ischaemia followed by 120 minutes of reperfusion. No further interventions were performed in the controls. IPC was elicited by 2 cycles of 5 minutes of global ischaemia interspersed with 5 minutes reperfusion prior to regional ischaemia. TNFα (0.5ng/ml) was given for 7 minutes followed by 10 minutes of reperfusion washout before the regional ischaemia. DDTC and SA were given for 12 minutes, breaching the period of drug treatment with 2,5 minutes of antagonise pre-post TNFα administration (TNFα/DDTC and TNFα/SA).

Figure 2

Schematic representation of the protocols used in the isolated perfused heart study. Infarct size was measured following the 120 minutes of reperfusion.

Fig. 2

Perfusion protocol for the isolated rat heart



13. Measurement of Risk Zone and Infarct Size

Infarct size and area at risk were determined as done previously.[72] At the end of the experiment, the silk suture around the coronary artery was securely tied and 0.7 ml of a 2% Evans Blue suspension was slowly infused through the aorta to delineate the myocardial risk zone from the non-compromised zone. The hearts were then frozen overnight before being cut into 2-mm thick slices (four to five slices per heart), defrosted, and stained by incubation for 5 minutes in 1% w/v triphenyltetrazolium chloride (TTC) in phosphate buffer. Estimation of the infarct size was accomplished by means of planimetric comparison of myocardium, which had lost activity of the hydrogenase enzyme system with myocardium devoid of direct coronary artery perfusion. Tetrazolium dyes formed coloured precipitates in the presence of the intact dehydrogenase enzyme systems. Hence, viable myocardium was delineated by a bright red appearance, whereas areas of myocardial necrosis lacked dehydrogenase activity and therefore was devoid of staining. The necrotic areas were distinctly discernible and thus quantifiable. Slices were then fixed in 10% v/v formaldehyde solution to enhance the contrast between stained viable tissue and unstained necrotic tissue. The area at risk and the area of infarcted tissue in the risk zone were determined using computerised planimetry (Summa Sketch III; Summa Graphics).

14. Phosphate Buffer for TTC Staining

The phosphate buffer is made up of 2 solutions. Solution 1 is 100mM Monobasic Sodium Phosphate (acidic): 15.6 g Na2PO4.2H2O in 1 L dH2O or 13.8 g $NaH_2PO_4.H_2O$ in 1 L dH_2O. Solution 2 is 100mM Dibasic Sodium Phosphate (alkaline): 14.2 g Na_2HPO_4 in 1L dH2O. The Phosphate buffer is 8 parts of solution 2 + 2 parts of solution 1. 250 mg of TTC is added to 25 ml of phosphate buffer to make the staining buffer.

15. Drugs

Diethyldithiocarbamate (DDTC), Sodium Salicylate (SA), Triphenyltetrazolium (TTC), Evans Blue (Direct Blue) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TNFα was purchased from PeproTech Inc Rocky Hill, NJ. TNFα was aliquated into 10 μg/ml stocks and kept frozen. Fresh TNFα was used for all experiments from frozen stocks. TNFα stability is fairly robust, however fresh stocks are used on a daily basis due to progressive degradation at room temperature. 5HD was made up fresh for each new experiment. Final concentration of 100μM of 5HD was prepared either with normoxic media / hypoxic media when it was used in cell culture experiments or with Krebs-Henselet buffer for the Langendorff perfusions.

16. Statistical Analysis

All data are presented as mean±S.E.M. Physiological variables were compared before and during coronary occlusion. The one-way analysis of

variance (ANOVA) was used to determine significance between groups in the metabolic studies and signalling studies. A P-value < 0.05 was considered statistically significant.

C. RESULTS

C. RESULTS

1. Simulated Ischaemia

The first objective was to establish a simulated ischaemia protocol in both cell lines. As mentioned before, the C2C12 cells are more susceptible to ischaemic damage when they reach the mature myotube stage. We used 8-10 days post differentiated C2C12 myotubes in all the experiments. The Girardi cells were used at a confluency of 100%. Simulated ischaemia was initiated by incubating the cells with a hypoxia buffer at pH 6.4 in hypoxic chamber (5% CO_2, 95% N_2). 8 hours ischaemia resulted in significant damage to the cells.

Fig. 3.1, Fig 3.2 and *Fig 3.3* show the ischaemic insult on the C2C12 and Girardi cells. Measurement of Cell viability was evaluated by spectrophotometrical measurement of LDH and via the measurement of propidium iodide uptake on flow cytometry. The more severe the cell damage the higher the release of LDH into the media and higher the propidium iodide (PI) uptake. The LDH and PI data has been normalised to ischaemic control = 100% percent damage value. This was done to enable comparisons across multiple experiments over a long time period. The 8 hour ischaemic insult causes around 60% increase in LDH release in both cell lines. The normalised data shows the normoxic values at 45±4 and 31±1 for the C2C12 and Girardi cells respectively. Concomitantly the PI data for the Girardi cells shows that there is a 95% increase in PI positive cells in response to 8 hour of simulated ischaemia.

Figure 3.1

Effect of an ischaemic insult (8 hours) on lactate dehydrogenase (LDH) release in C2C12 myotubes. Data is normalised to the ischaemic insult group (100%; $n \geq 6$).

Fig 3.1

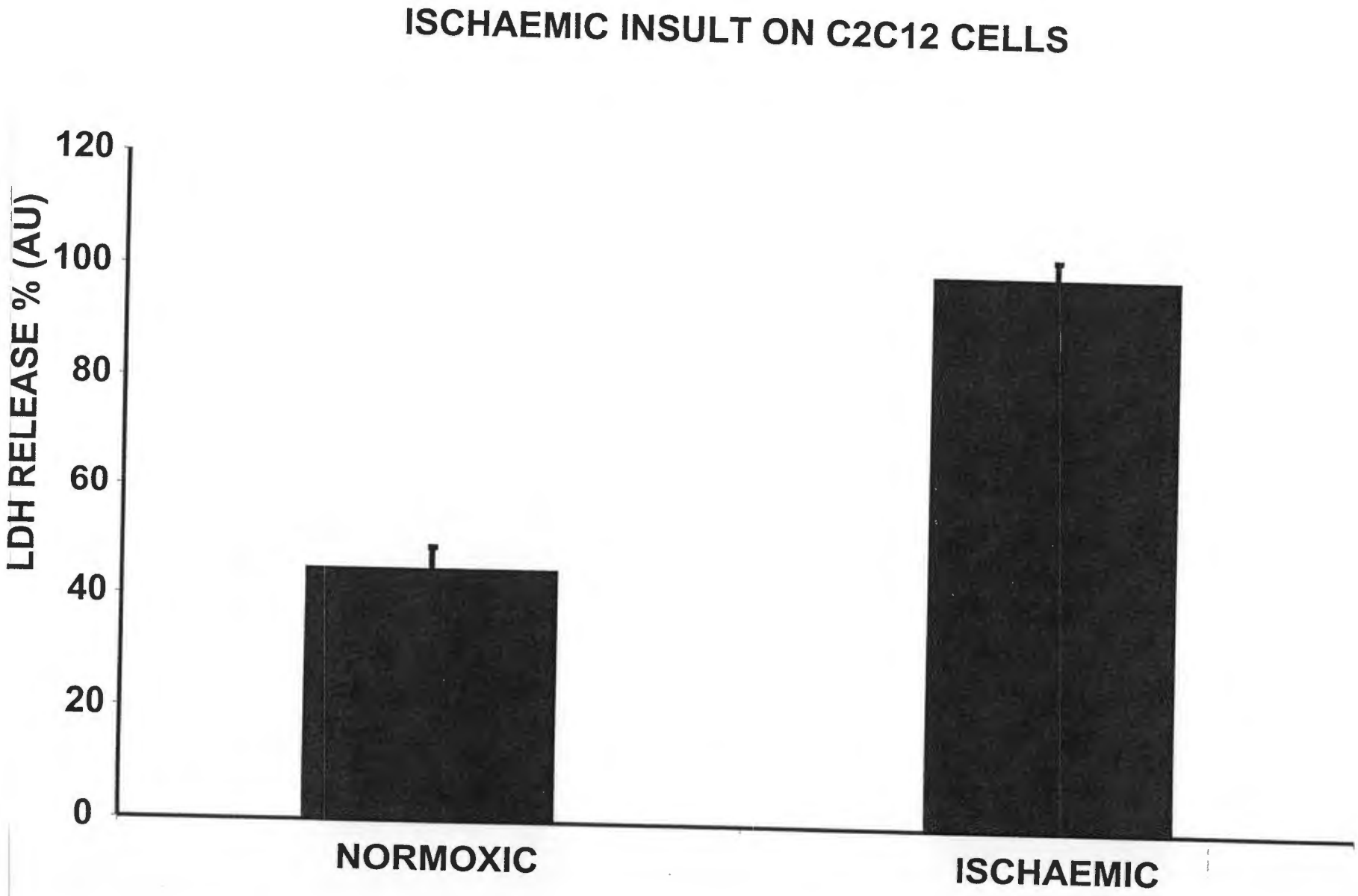

ISCHAEMIC INSULT ON C2C12 CELLS
LDH RELEASE % (AU)
120
100
80
60
40
20
0
NORMOXIC
ISCHAEMIC

Figure 3.2

Effect of an ischaemic insult (8 hours) on lactate dehydrogenase (LDH) release in Girardi cells. Data is normalised to the ischaemic group (100%; $n \geq 6$).

Fig 3.2

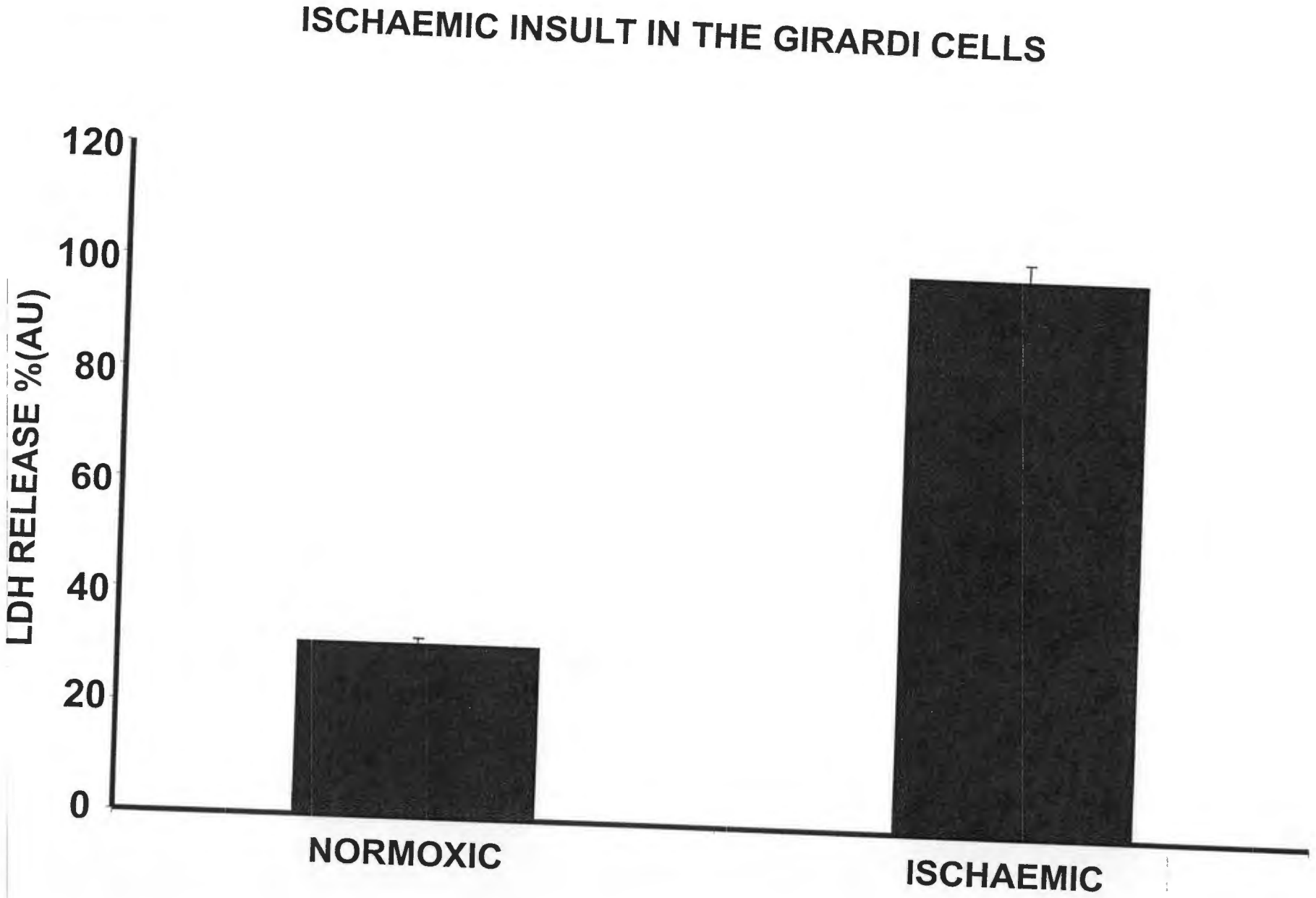
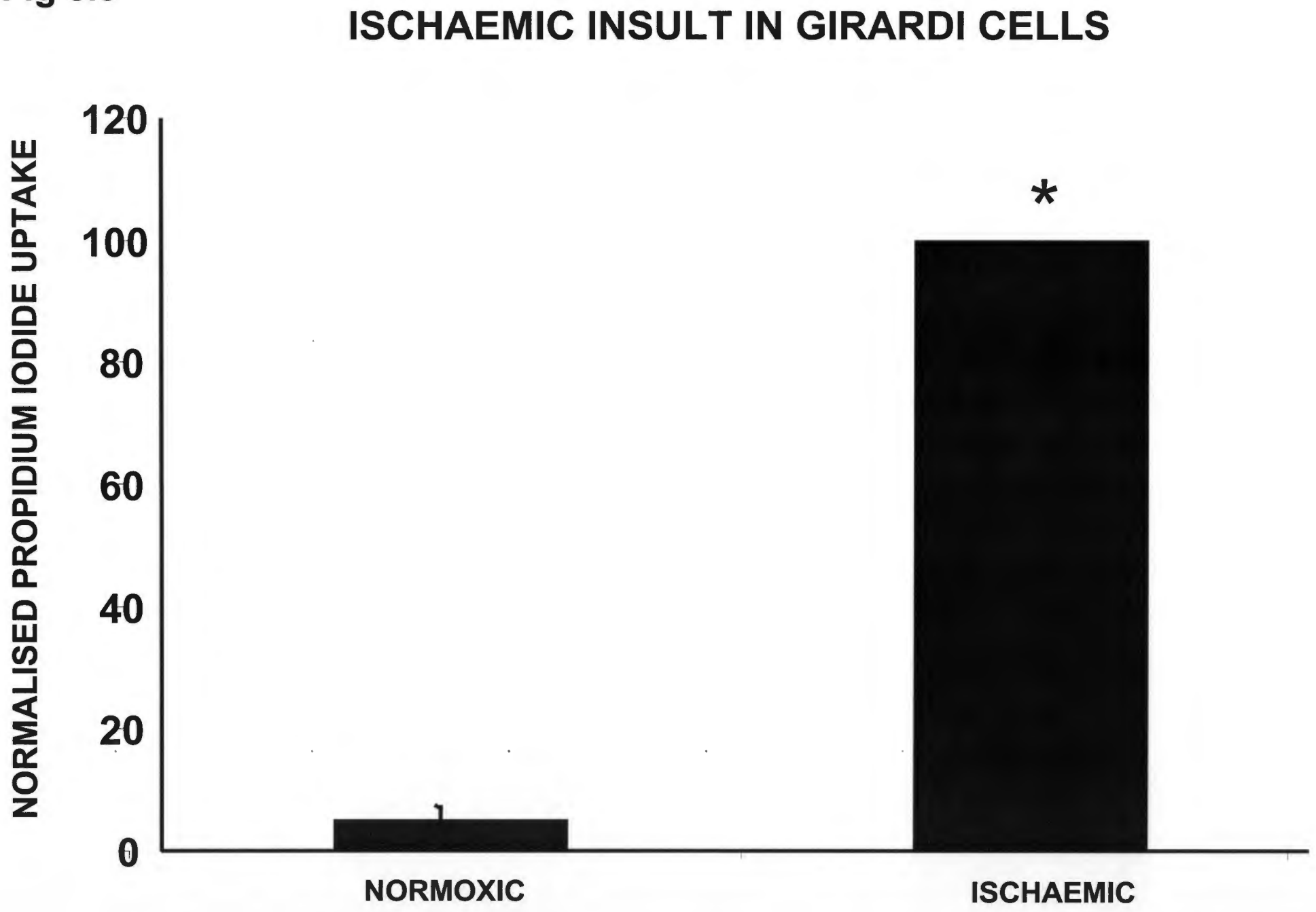

ISCHAEMIC INSULT IN THE GIRARDI CELLS
LDH RELEASE %(AU)
120
100
80
60
40
20
0
NORMOXIC
ISCHAEMIC

Figure 3.3

Effect of an ischaemic insult (8 hours) on propidium iodide (PI) positive cells. Data is normalised to the ischaemic group (100%; n=6 $p<0.001$)

Fig 3.3


ISCHAEMIC INSULT IN GIRARDI CELLS
NORMALISED PROPIDIUM IODIDE UPTAKE
120
100
80
60
40
20
0
*
NORMOXIC
ISCHAEMIC

2. Ischaemic Preconditioning in C2C12 and Girardi

Previous reports have shown that preconditioning could be cell-type specific.[63] Our second objective was to investigate whether the two cell lines, could be preconditioned, using the system described in the Method Section. Here, preconditioning was achieved by exposing the cell to a sublethal ischaemic insult and reoxygenation prior to a sustained ischaemic insult. The optimal preconditioning protocol was slightly different in each cell line. The slightly different protocol confirms Marban´s findings on cell-type specificity of preconditioning. The Girardi cells were more susceptible to ischaemic damage. The trigger ischaemic period was only 30 minutes compared to the 60 minutes of the C2C12 cells. However a 60 minute reperfusion/normoxic period prior to the eight hours ischaemia was applied to both. The trigger ischaemia buffer pH was slightly lower than the index ischaemia (eight hours) buffer. We used an ischaemia buffer of pH 6.2. *Fig. 4.1, Fig. 4.2* and *Fig 4.3* illustrate that the C2C12 and Girardi cells can be preconditioned using the respective protocols. IPC reduced cell damage (LDH release) by nearly 50%. LDH release was 53±3 and 57±1 % respectively for the C2C12 and Girardi cells. Our preconditioning protocol achieved the desired cytoprotection. There was, however, a bigger difference in the LDH release in the Girardi cells than in the C2C12 cells. As a confirmatory experiment, PI exclusion was assessed in the Girardi cells. As shown in Figure 4.3 the ischaemic preconditioning protocol attenuated PI uptake by 28±5% vs ischaemic controls.

Figure 4.1

Effect of ischaemic preconditioning (IPC) on lactate dehydrogenase (LDH) release following an ischaemic insult (8 hours) in C2C12 myotubes. Data is normalised to the ischaemic insult group (100%; $n \geq 6$; * $p<0.05$).

Fig 4.1

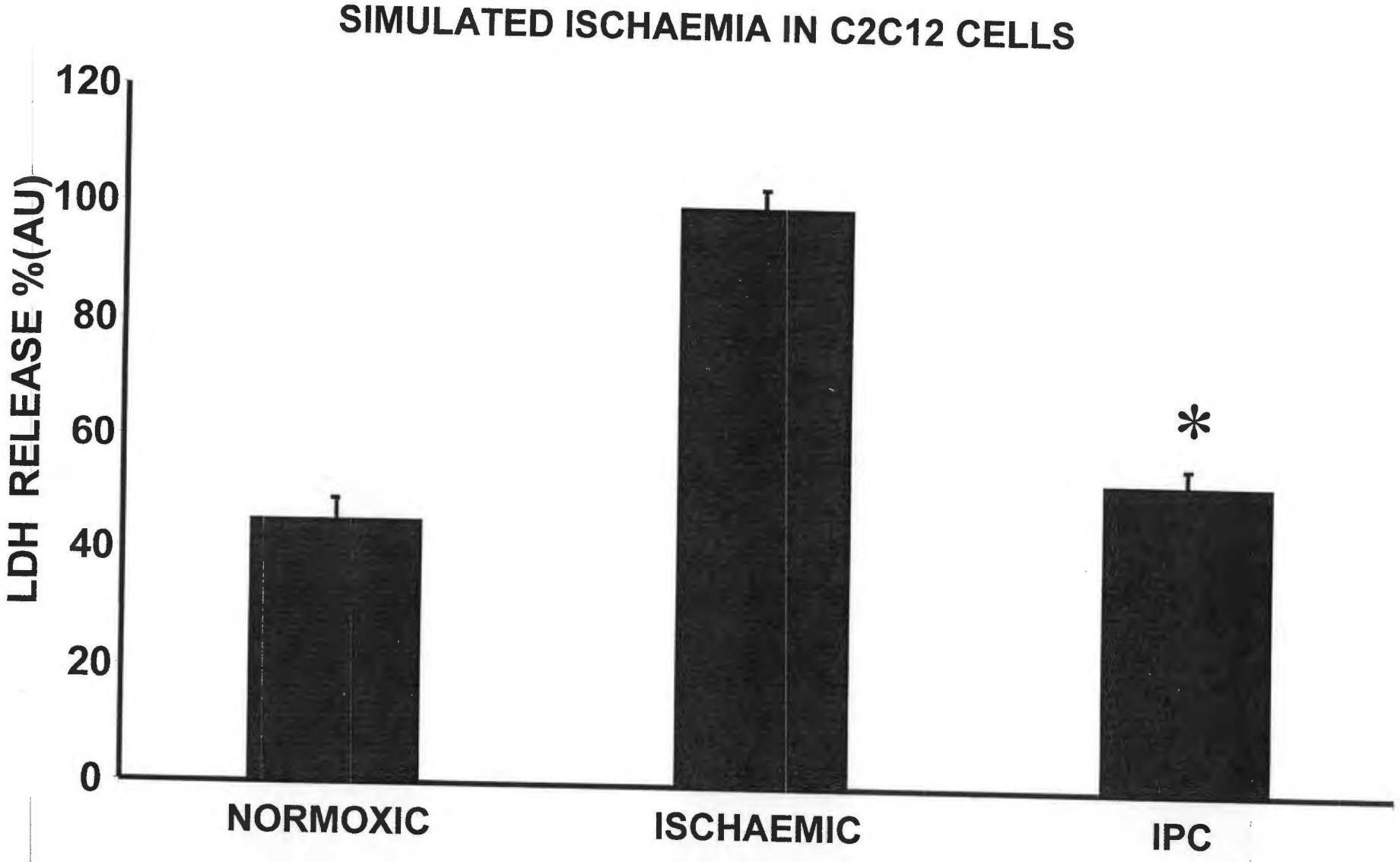

SIMULATED ISCHAEMIA IN C2C12 CELLS
LDH RELEASE %(AU)
120
100
80
60
40
20
0
*
NORMOXIC
ISCHAEMIC
IPC

Figure 4.2

Effect of ischaemic preconditioning (IPC) on lactate dehydrogenase (LDH) release following an ischaemic insult (8 hours) in Girardi cells. Data is normalised to the ischaemic insult group (100%; $n \geq 6$; * $p<0.05$).

Fig 4.2

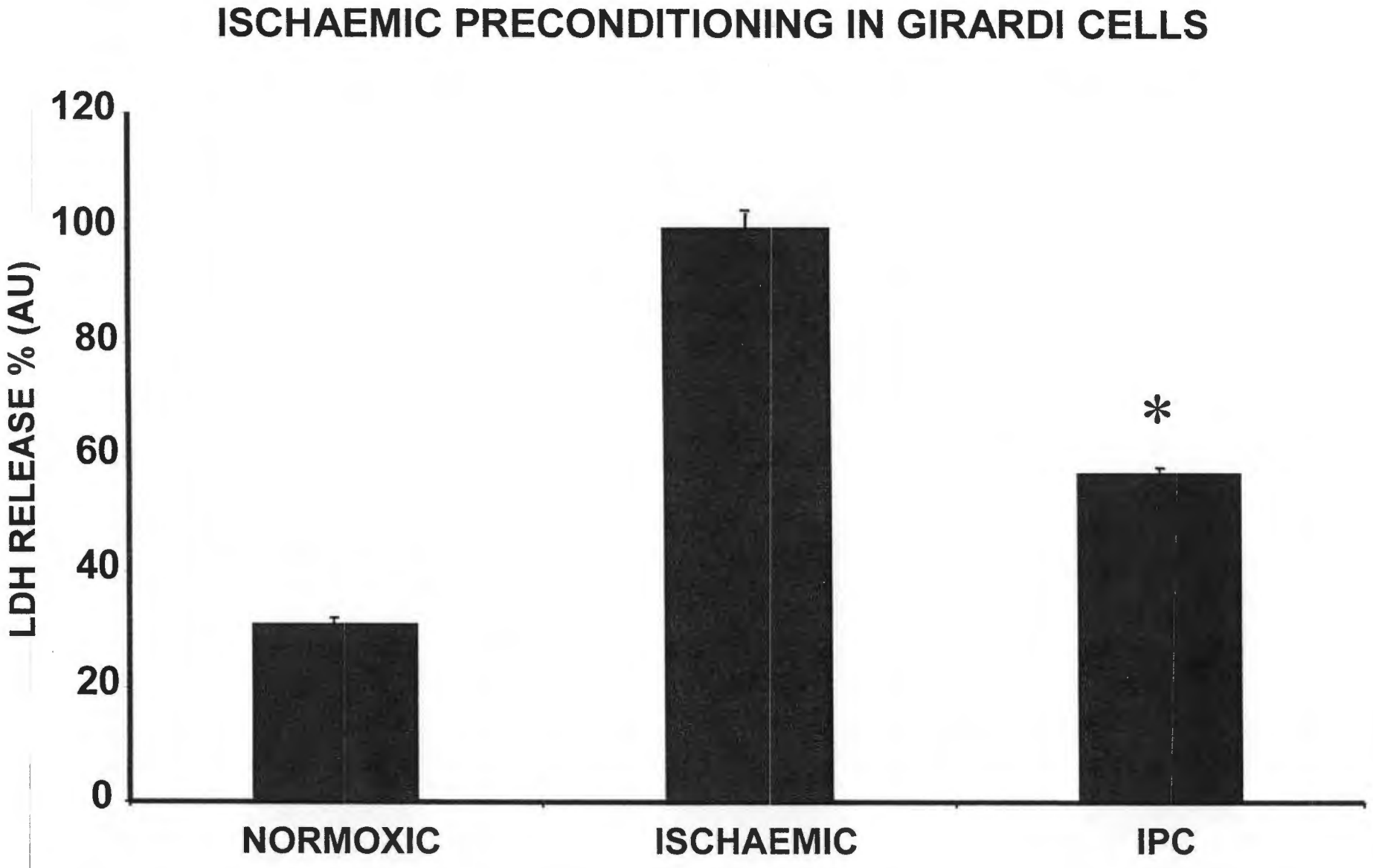
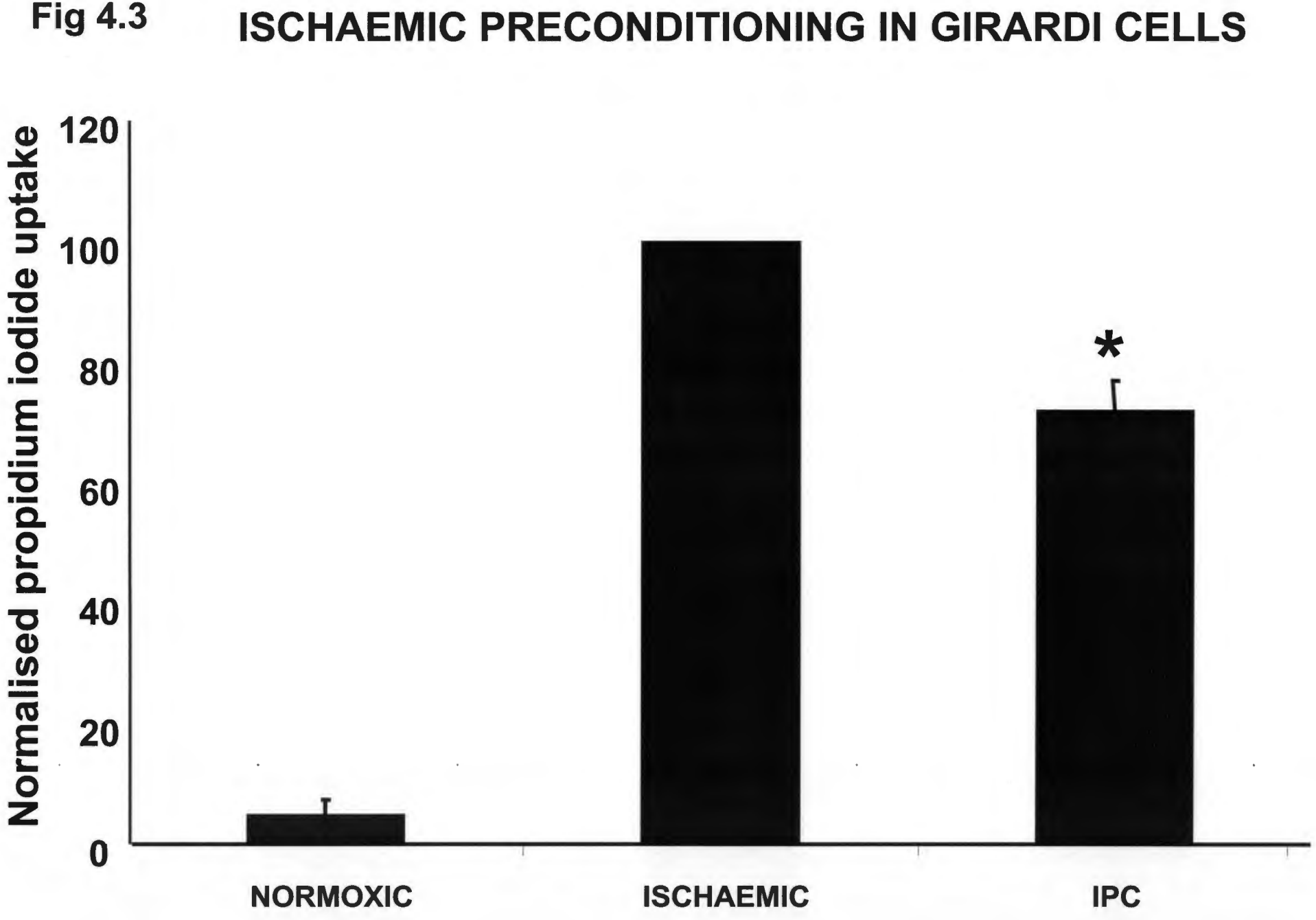

ISCHAEMIC PRECONDITIONING IN GIRARDI CELLS
LDH RELEASE % (AU)
120
100
80
60
40
20
0
*
NORMOXIC
ISCHAEMIC
IPC

Figure 4.3

Effect of ischaemic preconditioning (IPC) on propidium iodide (PI) positive cells following an ischaemic insult (8 hours) in Girardi cells. Data is normalised to the ischaemic insult group (100%, n=6; *$p<0.05$ vs ischaemic control

Fig 4.3


ISCHAEMIC PRECONDITIONING IN GIRARDI CELLS
Normalised propidium iodide uptake
120
100
80
60
40
20
0
*
NORMOXIC
ISCHAEMIC
IPC

3. Attenuation of IPC in C2C12 and Girardi Cells

The preconditioning phenomenon has been described for the last 14 years. The development of specific and non specific blockers of the preconditioning programme has been identified. 5-hydoxydecanoic acid (5HD) is one of the most used preconditioning blockers. 5HD is a specific blocker of the mitochondrial K_{ATP} channel. Mitochondrial K_{ATP} channels are thought to be pivotal effectors in the protective cascade induced by preconditioning. 5HD has been shown to block IPC in various species. *Fig 5.1* and *Fig 5.2* demonstrate the effect of the known preconditioning blockers on the IPC groups in both cell lines. Adenosine, a known preconditioning agent, could also precondition the Girardi cells. The preconditioning blockers attenuated the cytoprotection of IPC in both cell lines. The 100μM 5HD does not fully block/inhibit the protective effect. In previous studies we found that 1 mM was toxic to the cells. Glibenclamide blocks both sarcolemmal and mitochondrial K_{ATP} channels, also attenuated the protection when used with the C2C12 cells. Our results thus confirm that classical preconditioning is possible in our two cell lines and that the cytoprotection achieved through our protocol might follow the signalling pattern that is widely accepted at the moment. Although the underlying mechanism of ischaemic preconditioning is still not fully understood, evidence obtained through pharmacological studies suggests that adenosine receptors, protein kinase C and K_{ATP} channels are involved.

Figure 5.1

Effect of the K_{ATP} channel blockers glibenclamide (100 μM) and 5-hydroxydecanoate (5HD; 100μM) on ischaemic preconditioning (IPC) as measured by lactate dehydrogenase (LDH) release following an ischaemic insult (8 hours) in C2C12 myotubes. Data is normalised to the ischaemic insult group (100%; $n \geq 6$; * $p<0.05$ vs Ischaemic; # $p<0.05$ vs IPC).

Fig 5.1

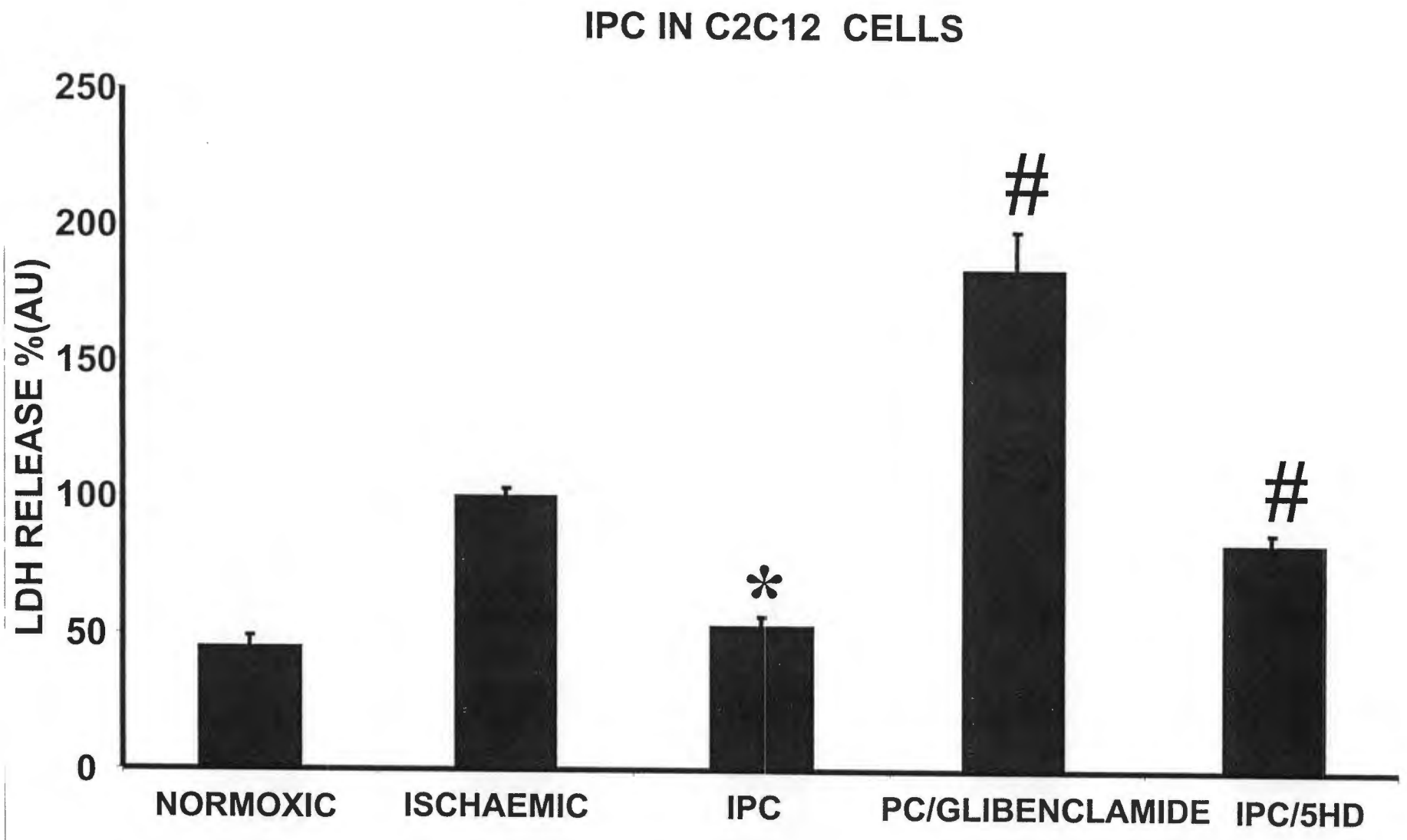
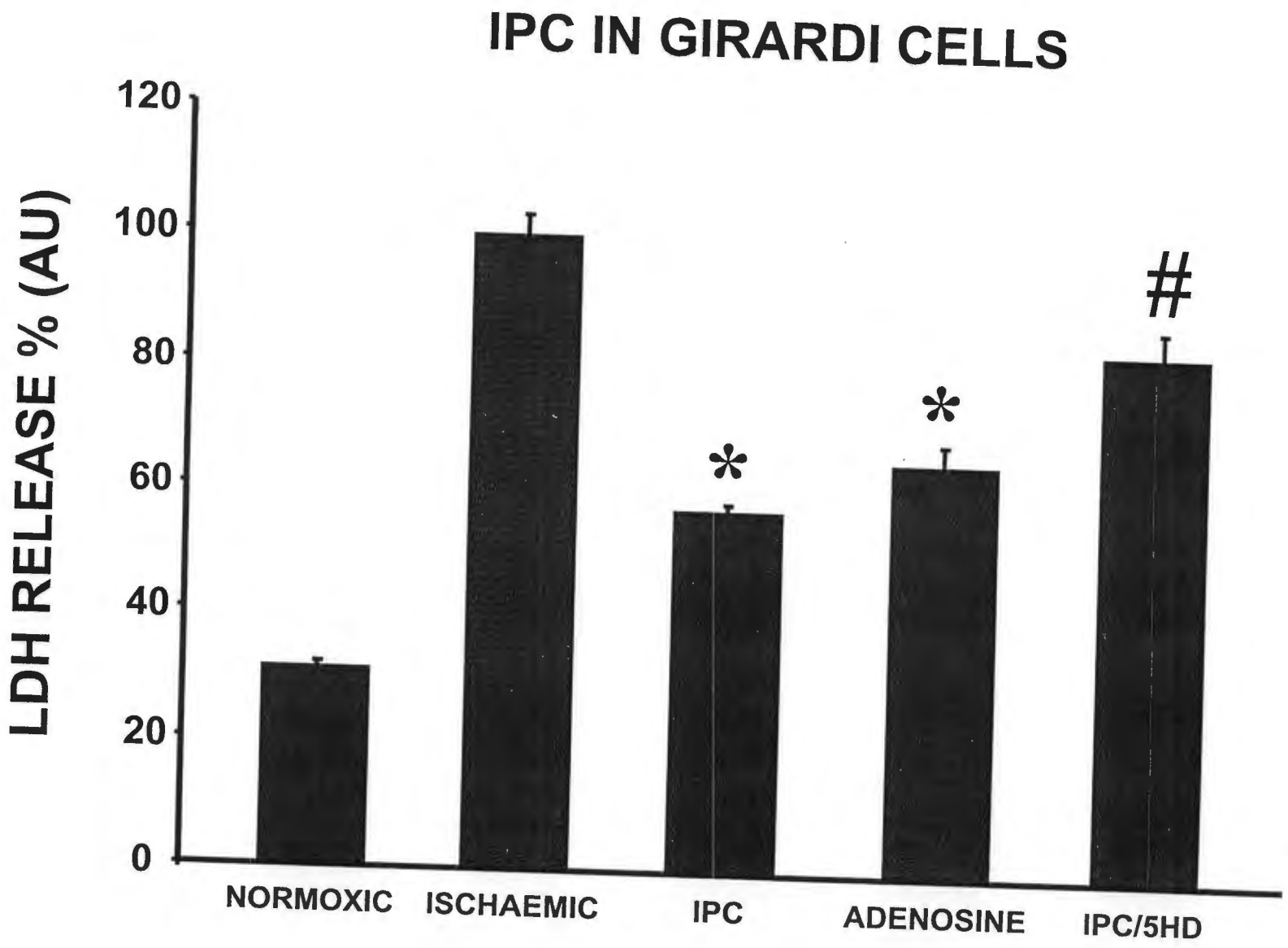

IPC IN C2C12 CELLS
LDH RELEASE %(AU)
250
200
150
100
50
0
*
#
#
NORMOXIC
ISCHAEMIC
IPC
PC/GLIBENCLAMIDE
IPC/5HD

Figure 5.2

Effect of the mitochondrial KATP channel blocker 5-hydroxydecanoate (5HD; 100 μM) on ischaemic preconditioning (IPC) and the effect of pre-treatment with adenosine (100 μM) as measured by lactate dehydrogenase (LDH) release following an ischaemic insult (8 hours) in Girardi cells. Data is normalised to the ischaemic insult group (100%; $n \geq 6$; * $p<0.05$ vs Ischaemic; #$p< 0.05$ vs IPC).

Fig 5.2


IPC IN GIRARDI CELLS
LDH RELEASE % (AU)
120
100
80
60
40
20
0
*
*
#
NORMOXIC
ISCHAEMIC
IPC
ADENOSINE
IPC/5HD

4. TNFα as a PC mimetic Agent

After establishing a cellular model of preconditioning with the two cell types, we then investigated the temporal and dose requirements of TNFα in promoting protection against ischaemic injury. TNFα was initially used as a preconditioning mimetic using a dose ranging from 0.005 ng/ml to 20 ng/ml in both cell lines. The PC mimetic protocol for TNFα was similar to the IPC protocol for both cell line respectively (see *Fig 1.1* and *Fig 1.2*). In *Fig 6.1* , *6.2* and 6.3 we clearly demonstrate that a 0.5 ng/ml TNFα is cytoprotective for both cell lines. TNFα induced cytoprotection(LDH release) by nearly 40% in both cell lines compared to ischaemic controls. Using propidium iodide exclusion as an additional index of cell viability, we have confirmed that the optimal dose of TNFα does attenuates PI uptake by 42±5% vs ischaemic controls ($p<0.001$) when TNFα was administered as a preconditioning mimetic

Figure 6.1

Effect of recombinant TNFα (0.5 ng/ml) pre-treatment (1 hour exposure followed by 1 hour wash off) on lactate dehydrogenase (LDH) release in C2C12 myotubes following an ischaemic insult (8 hours). Data is normalised to the ischaemic insult group (100%; $n \geq 6$; * $p<0.05$ vs Ischaemic).

Fig 6.1

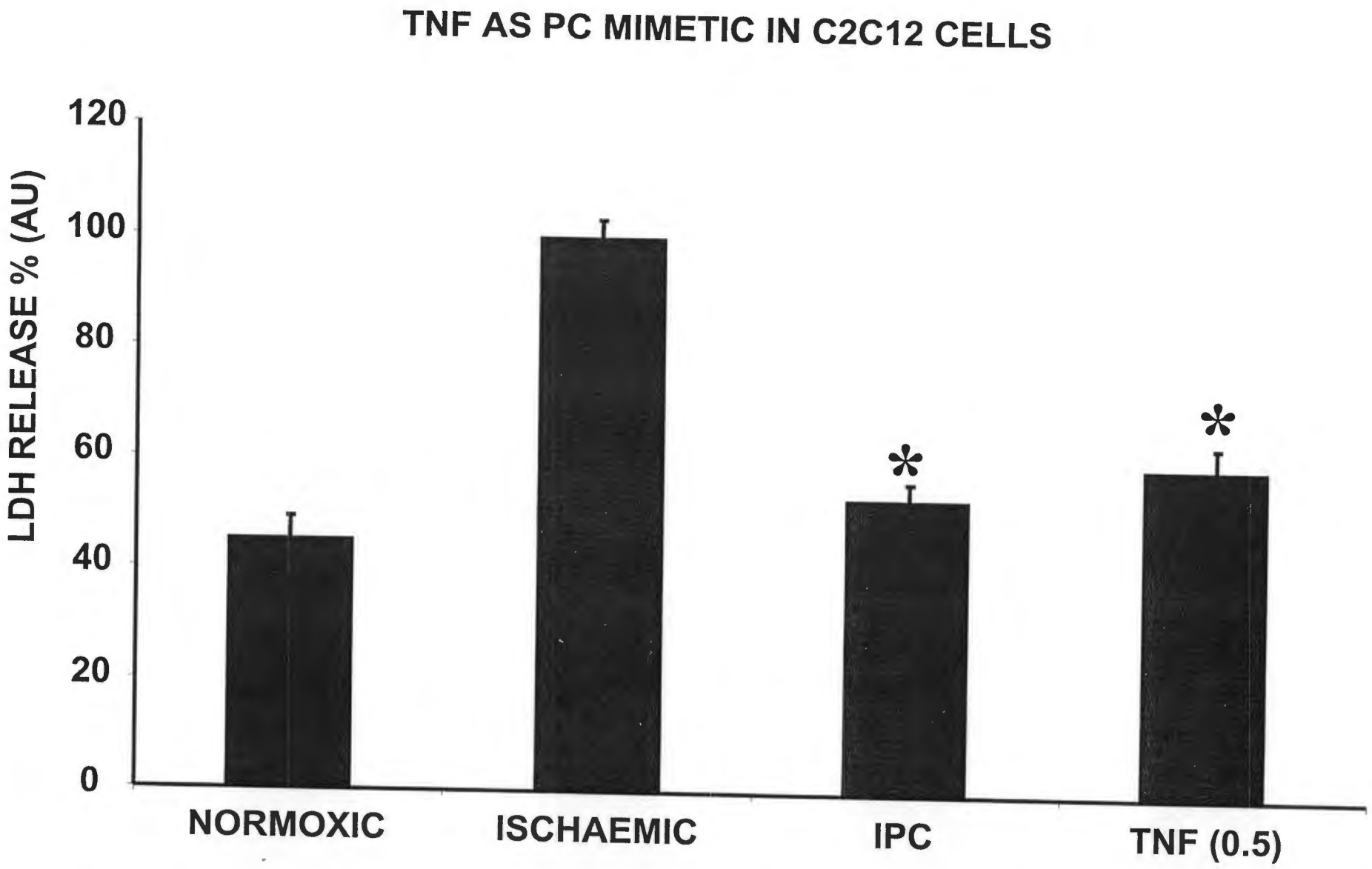

TNF AS PC MIMETIC IN C2C12 CELLS
LDH RELEASE % (AU)
120
100
80
60
40
20
0
*
*
NORMOXIC
ISCHAEMIC
IPC
TNF (0.5)

Figure 6.2

Effect of recombinant TNFα (0.5 ng/ml) pre-treatment (30 minutes exposure followed by 1 hour wash off) on lactate dehydrogenase (LDH) release in Girardi cells following an ischaemic insult (8 hours). Data is normalised to the ischaemic insult group (100%; $n \geq 6$; * $p<0.05$ vs Ischaemic).

Fig 6.2

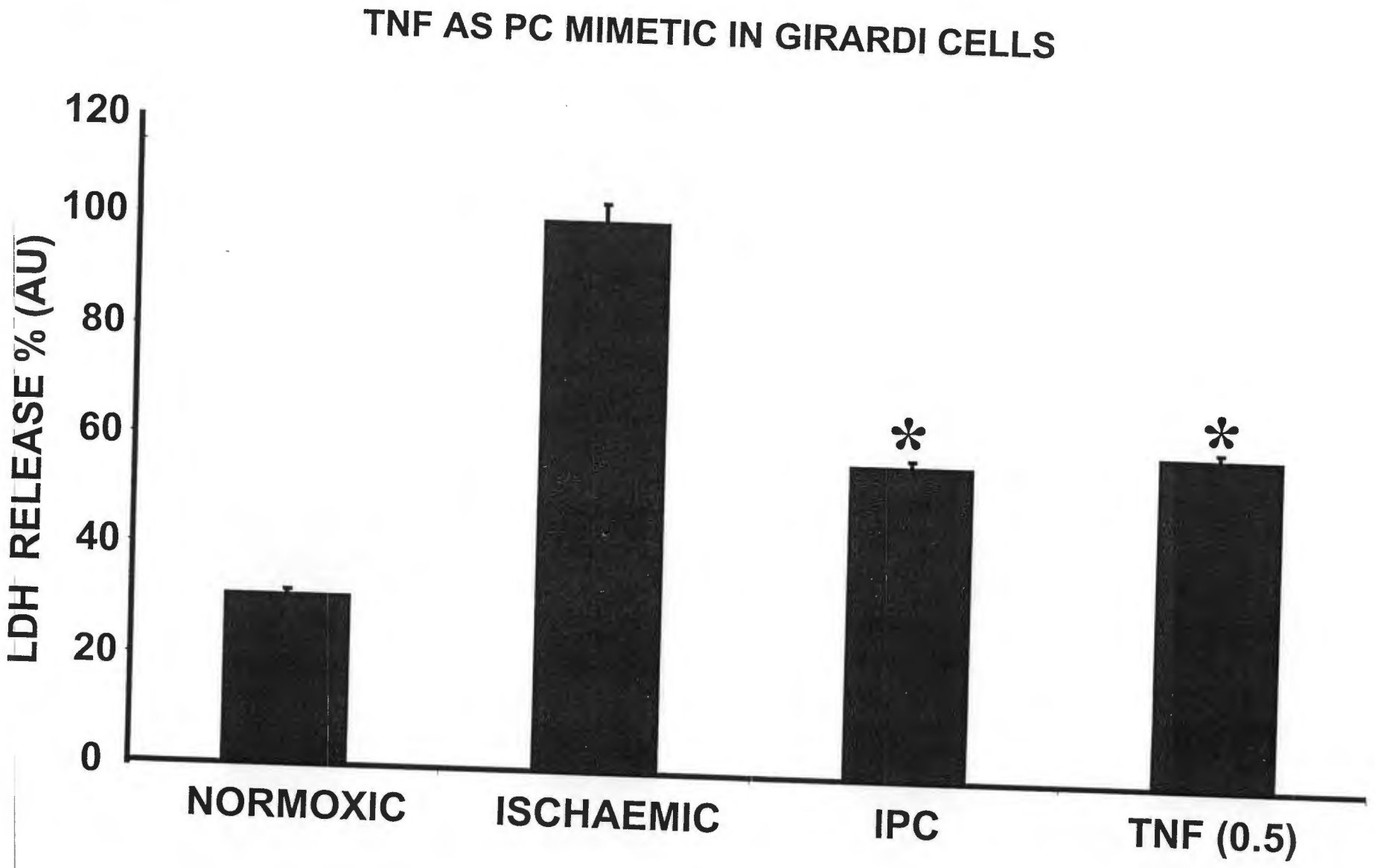
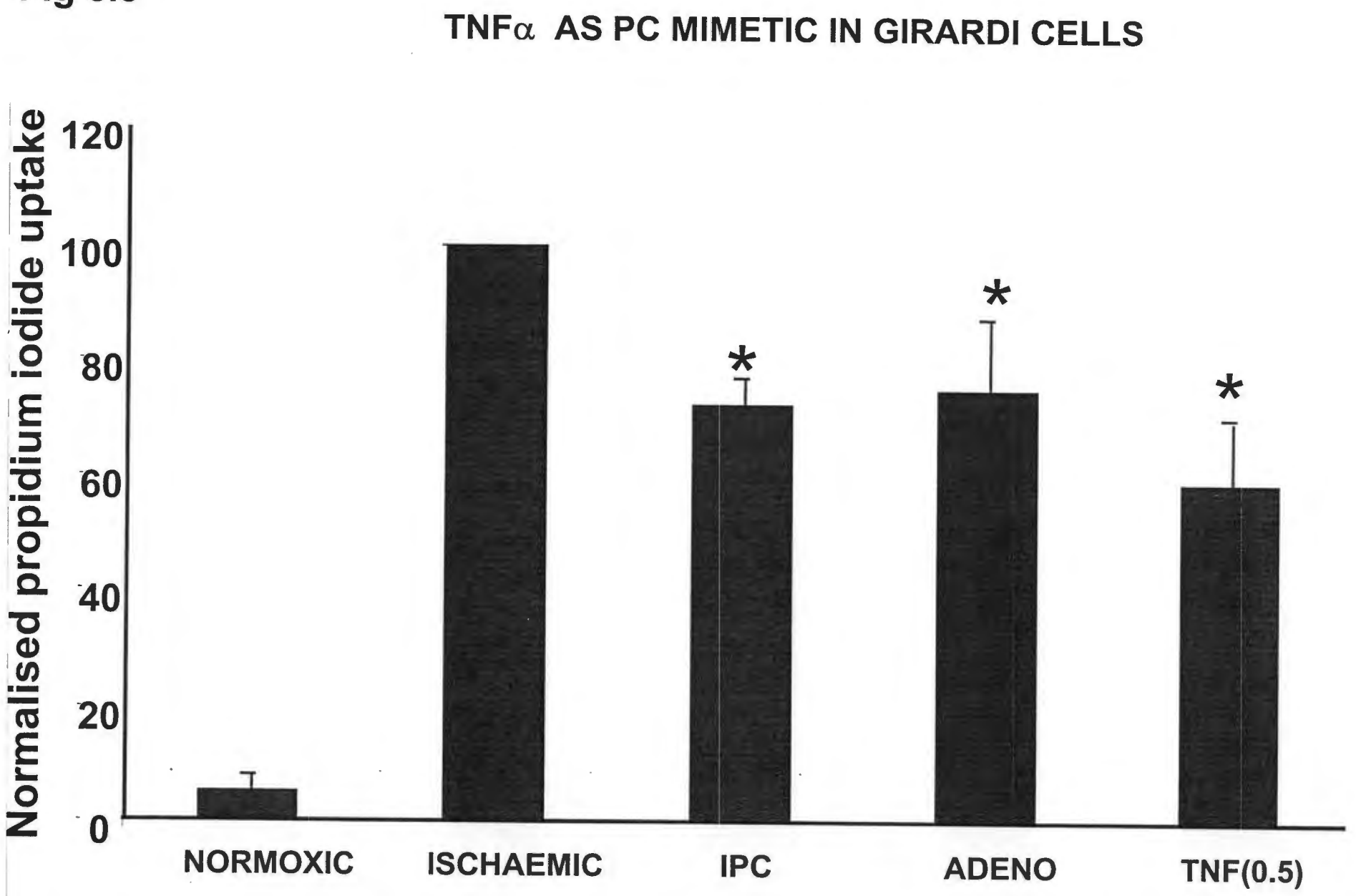

TNF AS PC MIMETIC IN GIRARDI CELLS
LDH RELEASE % (AU)
120
100
80
60
40
20
0
*
*
NORMOXIC
ISCHAEMIC
IPC
TNF (0.5)

Figure 6.3

Effect of recombinant TNFα (0.5 ng/ml) as a preconditioning-mimetic (1 hour exposure followed by 1 hour wash off) on propidium iodide (PI) positive cells in Girardi cells following an ischaemic insult (8 hours). Data is normalised to the ischaemic insult group (100%; n=6; *$p<0.05$ vs ischaemic controls)

Fig 6.3


TNFα AS PC MIMETIC IN GIRARDI CELLS
Normalised propidium iodide uptake
120
100
80
60
40
20
0
*
*
*
NORMOXIC
ISCHAEMIC
IPC
ADENO
TNF(0.5)

In *Fig 7.1* and *7.2* the dose response of TNFα on the two cells is presented. The cytoprotective doses of TNFα range from 0.005 to 0.5 ng/ml. Doses that were cardiotoxic ranged from 5.0 ng/ml up to 20 ng/ml. The transition from being cytoprotective to deleterious to the cell is between 0.5 ng/ml and 5 ng/ml. There is a 60% increase in LDH release between 0.5 ng/ml and 5.0 ng/ml. (0.5 ng/ml=60±1, 5.0 ng/ml=123±5). We did not go higher than 5 ng/ml with the C2C12 cells as the dose effect seen for 0.005-5.0-ng/ml mirrored that established in the Girardi cells. It is clearly evident that lower (physiological) doses of TNFα are cytoprotective in both these two cell lines. The higher (supraphysiological) doses are detrimental to the cell.

Figure 7.1

Effect of increasing dose of TNFα (0.005 – 5.0 ng/ml) pre-treatment (1 hour exposure followed by 1 hour wash off) on lactate dehydrogenase (LDH) release in C2C12 myotubes following an ischaemic insult (8 hours). Data is normalised to the ischaemic insult group (100%; $n \geq 6$; * $p<0.05$ vs Ischaemic).

Fig 7.1

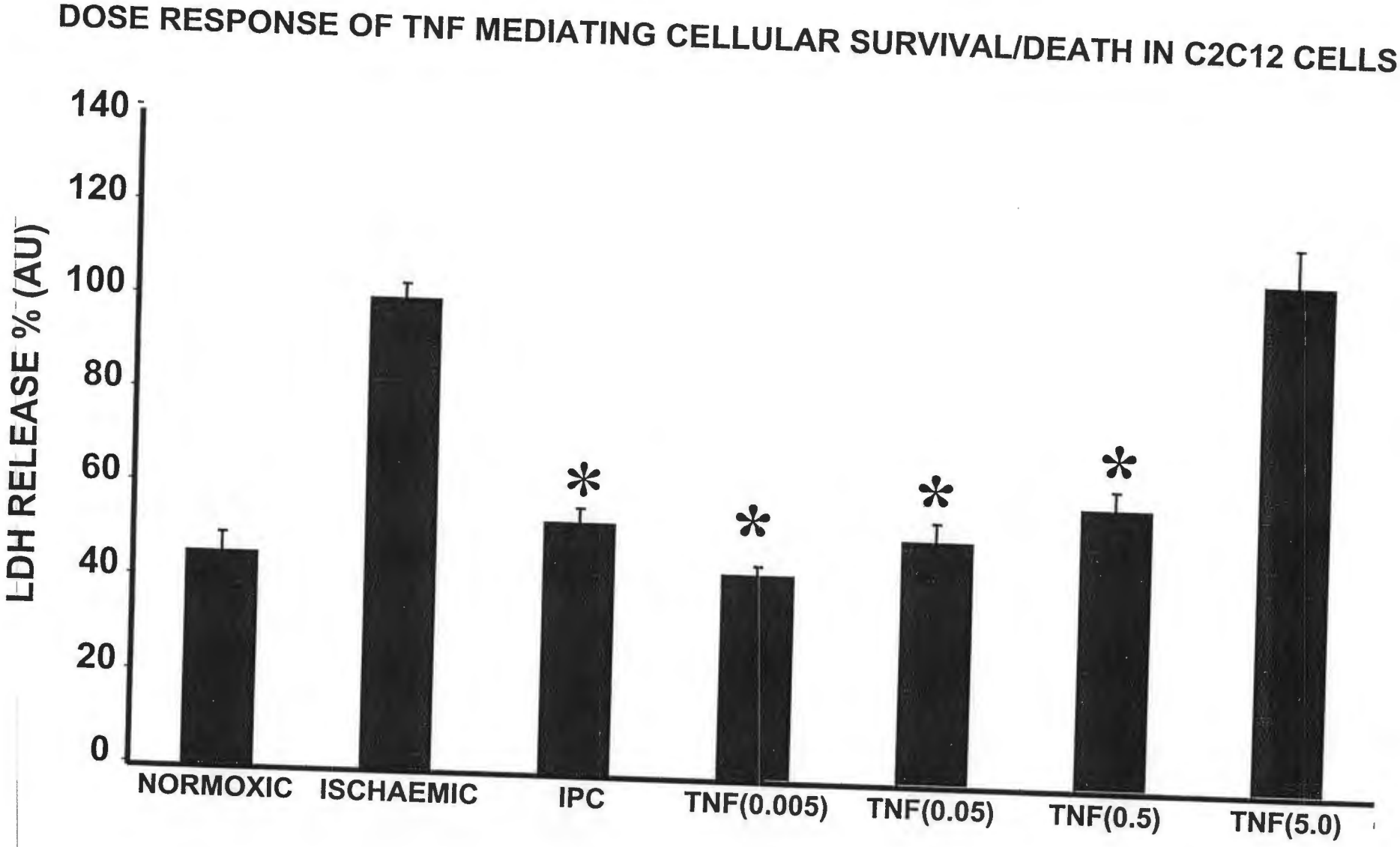
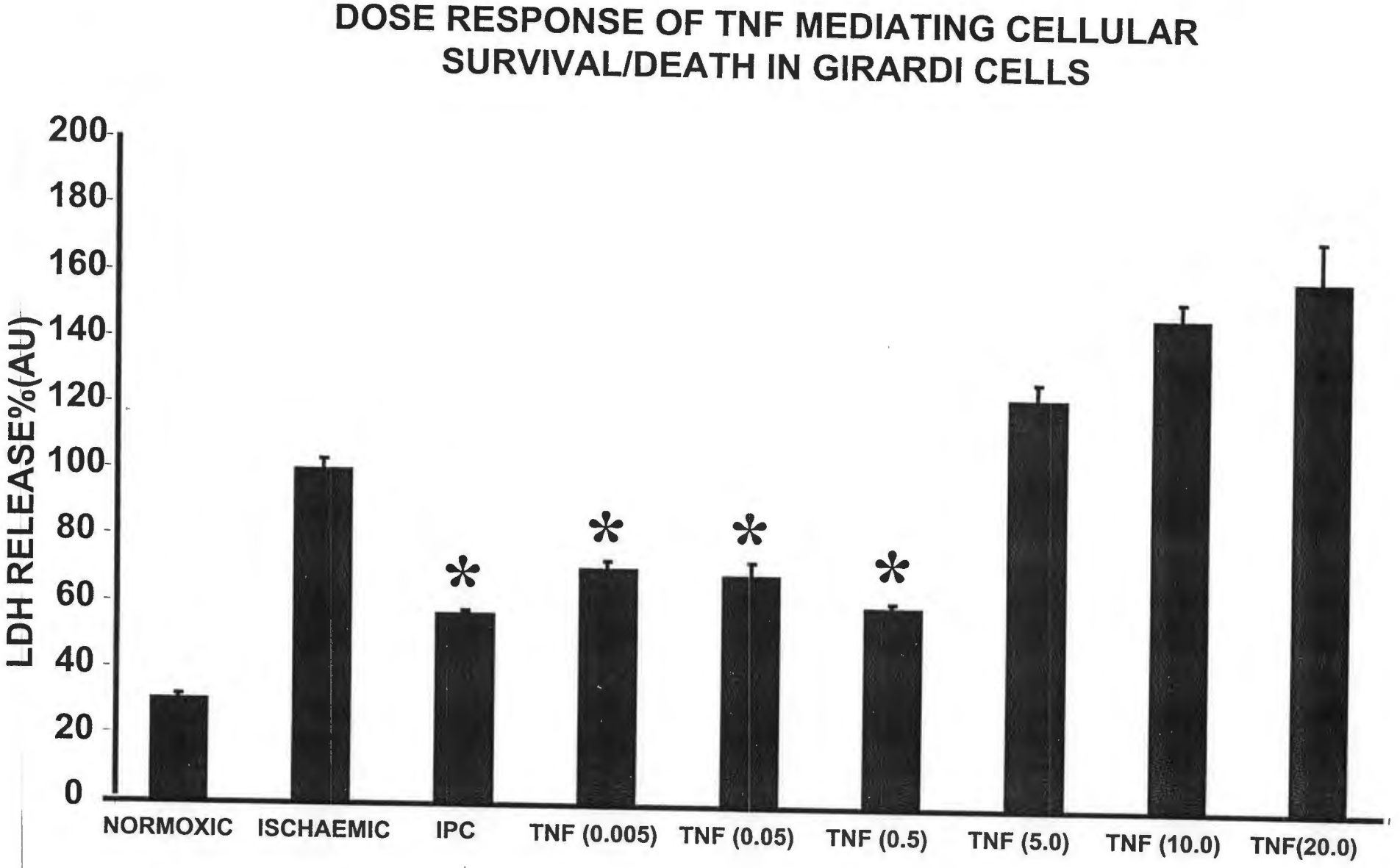

DOSE RESPONSE OF TNF MEDIATING CELLULAR SURVIVAL/DEATH IN C2C12 CELLS
LDH RELEASE % (AU)
140
120
100
80
60
40
20
0
*
*
*
*
NORMOXIC
ISCHAEMIC
IPC
TNF(0.005)
TNF(0.05)
TNF(0.5)
TNF(5.0)

Figure 7.2

Effect of increasing dose of TNFα (0.005 – 20,0 ng/ml) pre-treatment (30 minutes exposure followed by 1 hour wash off) on lactate dehydrogenase (LDH) release in Girardi cells following an ischaemic insult (8 hours). Data is normalised to the ischaemic insult group (100%; $n \geq 6$; *$p<0.05$ vs Ischaemic).

Fig 7.2


DOSE RESPONSE OF TNF MEDIATING CELLULAR SURVIVAL/DEATH IN GIRARDI CELLS
LDH RELEASE%(AU)
200
180
160
140
120
100
80
60
40
20
0
*
*
*
*
NORMOXIC
ISCHAEMIC
IPC
TNF (0.005)
TNF (0.05)
TNF (0.5)
TNF (5.0)
TNF (10.0)
TNF(20.0)

5. Temporal Effects of TNFα Administration

TNFα PC mimetic abilities afford cytoprotection in both cell lines. The next step was to investigate the temporal effects of this cytokine to see if it can protect the cells against ischaemic damage. For the temporal studies we incubated both cell lines with 0.5 ng/ml TNFα during the one hour reperfusion (pretreatment), before the eight hours ischaemic period as well as during the eight hours ischaemic period (hypoxia). *Fig. 8.1* and *Fig. 8.2* demonstrate the temporal effect of 0.5 ng/ml TNFα as seen with the two cell lines. In the Girardi cells there is a cytoprotective effect of TNFα in both pretreatment as well as in the eight hours ischaemic period. It seems that TNFα also has an anti-ischaemic property. In the C2C12 cells the TNFα does not have a cytoprotective effect, as the LDH release is nearly the same as the ischaemic control (91±5). The TNFα was also incubated with a normoxic control and had no appreciable effects. The unique cytoprotection confered by TNFα is clearly demonstrated here in *Fig 8.1* and *8.2*. However, there is no explanation for the detrimental effects of 0.5 ng/ml TNFα in the eight hours hypoxic/ischaemic period. Being a PC mimetic agent, pretreatment agent and an anti-ischaemic agent in these cells support the concept that TNFα may be favourable cytoprotective agent.

Figure 8.1.

Temporal effects of TNFα on lactate dehydrogenase (LDH) release in C2C12 myotubes following an ischaemic insult (8 hours). The treatment groups are as follows: TNFα (0.5 ng/ml) pre-treatment (1 hour exposure followed by 1 hour wash off); TNFα (0.5 ng/ml) pre-treatment (1 hour exposure wash off immediately prior to the ischaemic insult) and TNFα (0.5 ng/ml) exposure during the ischaemic insult. Data is normalised to the ischaemic insult group (100%; $n \geq 6$; *$p<0.05$ vs Ischaemic).

Fig 8.1

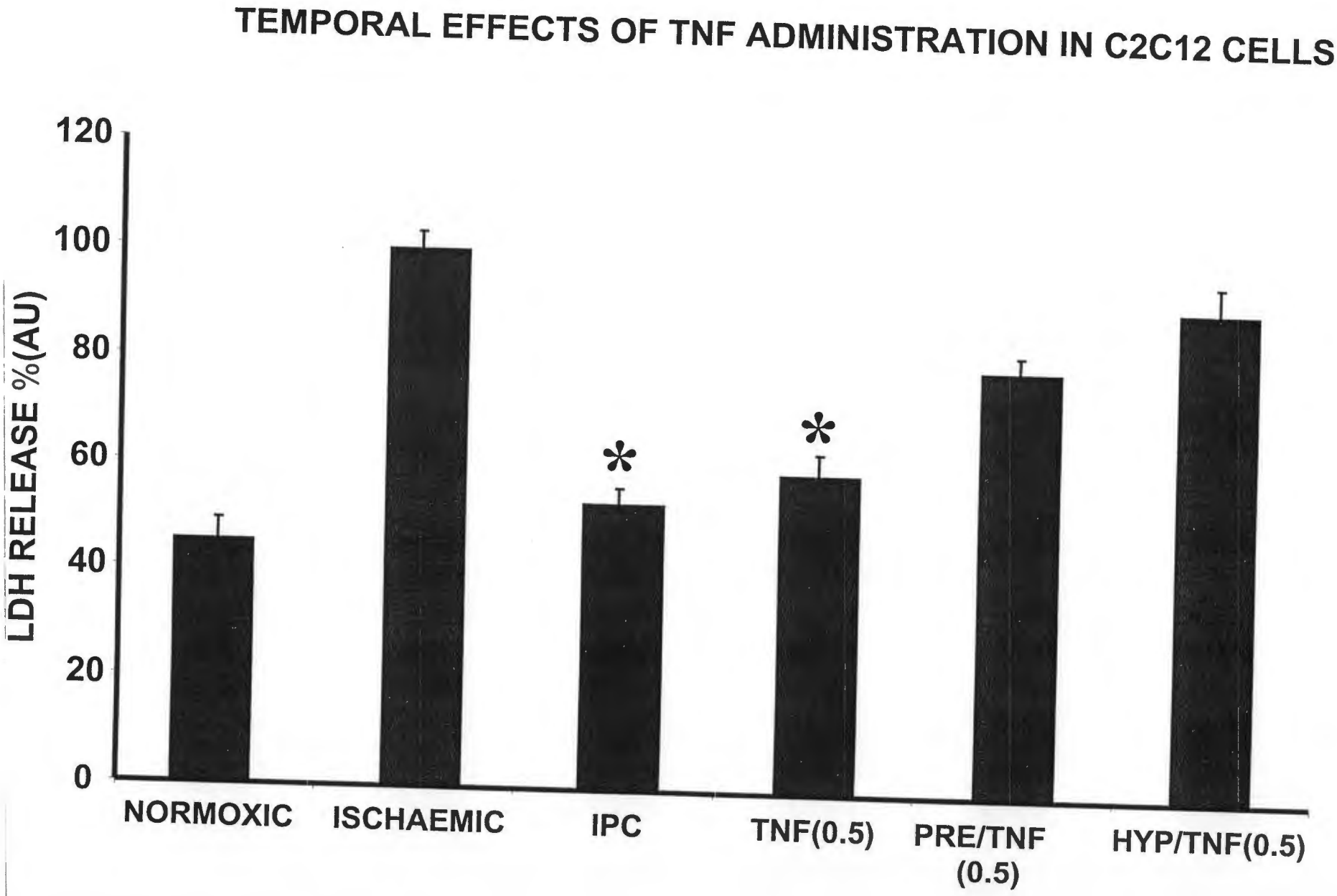
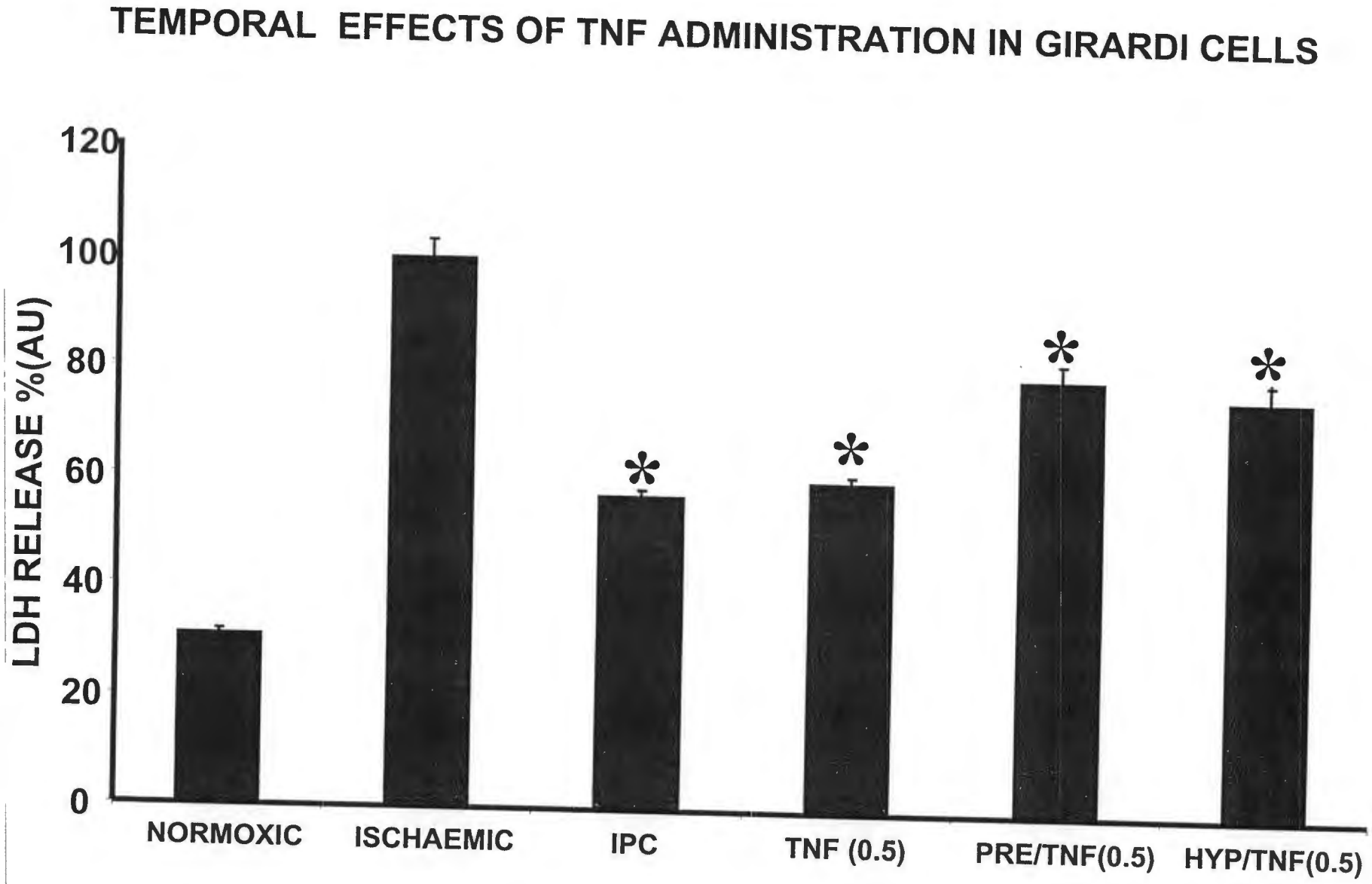

TEMPORAL EFFECTS OF TNF ADMINISTRATION IN C2C12 CELLS
LDH RELEASE %(AU)
120
100
80
60
40
20
0
*
*
NORMOXIC
ISCHAEMIC
IPC
TNF(0.5)
PRE/TNF (0.5)
HYP/TNF(0.5)

Figure 8.2

Temporal effects of TNFα on lactate dehydrogenase (LDH) release in Girardi cells following an ischaemic insult (8 hours). The treatment groups are as follows: TNFα (0.5 ng/ml) pre-treatment (30 minutes exposure followed by 1 hour wash off); TNFα (0.5 ng/ml) pre-treatment (1 hour exposure wash off immediately prior to the ischaemic insult) and TNFα (0.5 ng/ml) exposure during the ischaemic insult. Data is normalised to the ischaemic insult group (100%; $n \geq 6$; *$p<0.05$ vs Ischaemic).

Fig 8.2


TEMPORAL EFFECTS OF TNF ADMINISTRATION IN GIRARDI CELLS
LDH RELEASE %(AU)
120
100
80
60
40
20
0
*
*
*
*
NORMOXIC
ISCHAEMIC
IPC
TNF (0.5)
PRE/TNF(0.5)
HYP/TNF(0.5)

6. Attenuation of IPC and TNFα by NFκ B Antagonists

The cytoprotective ability of IPC has been studied extensively and is still ongoing. The elucidation of the signalling pathway involved in the cellular protection of IPC needs further investigation. It was recently being suggested that a transcriptional activator might be involved in the cytoprotection induced by IPC. Transcriptional activation might be an important step as it has been found that using agents like cyclohexamide and actinomycin D could abolish the cytoprotection of SWOP.[4] Bolli recently showed that the transcriptional activator NFκ B might be an important mediator in the signalling of IPC. He used a NFκ B blocker (DDTC) in his IPC experiments.[4] DDTC abolished the cytoprotection of IPC on experiments with rabbits. Thus, we investigated the signalling pathway involved in TNFα cytoprotection. As TNFα and IPC mediated more or less the same percentage of cytoprotection in our cellular model we proposed that they might share common signalling pathways in their protection. We then set up experiments by using two NFκ B blockers along with the IPC and TNFα group. The additional NFκ B blocker we used was sodium salicylate (SA).[73] The two blockers were incubated along with the IPC trigger and PC mimetic period respectively. In *Fig. 9.1* and *9.2* we can clearly demonstrate that the protection of both IPC and 0.5 ng/ml TNFα were attenuated by the two NFκ B blockers. This data suggest that the IPC and TNFα might share a common cytoprotective pathway. It is also shown that the cytoprotection is the same in both cell lines. It seems the transcriptional activator NFκ B plays an integral mediator role in the protection signalling system of IPC and TNFα.

Figure 9.1

Effect of NF-κB inhibition by salicylic acid (10 mM) and diethyldithiocarbamate (DDTC; 10 mM) on ischaemic preconditioning (1 hour simulated ischaemia and 1 hour reperfusion prior to the index ischaemia) and TNFα (0.5 ng/ml) pre-treatment (1 hour exposure followed by 1 hour wash off) on lactate dehydrogenase (LDH) release in C2C12 myotubes following an ischaemic insult (8 hours). Data is normalised to the ischaemic insult group (100%; $n \geq 6$; *$p<0.05$ vs Ischaemic; #$p<0.05$ vs IPC; +$p<0.05$ vs TNFα).

Fig 9.1

IPC AND TNF PC ARE ATTENUATED BY NF κB ANTAGONISTS IN C2C12 CELLS

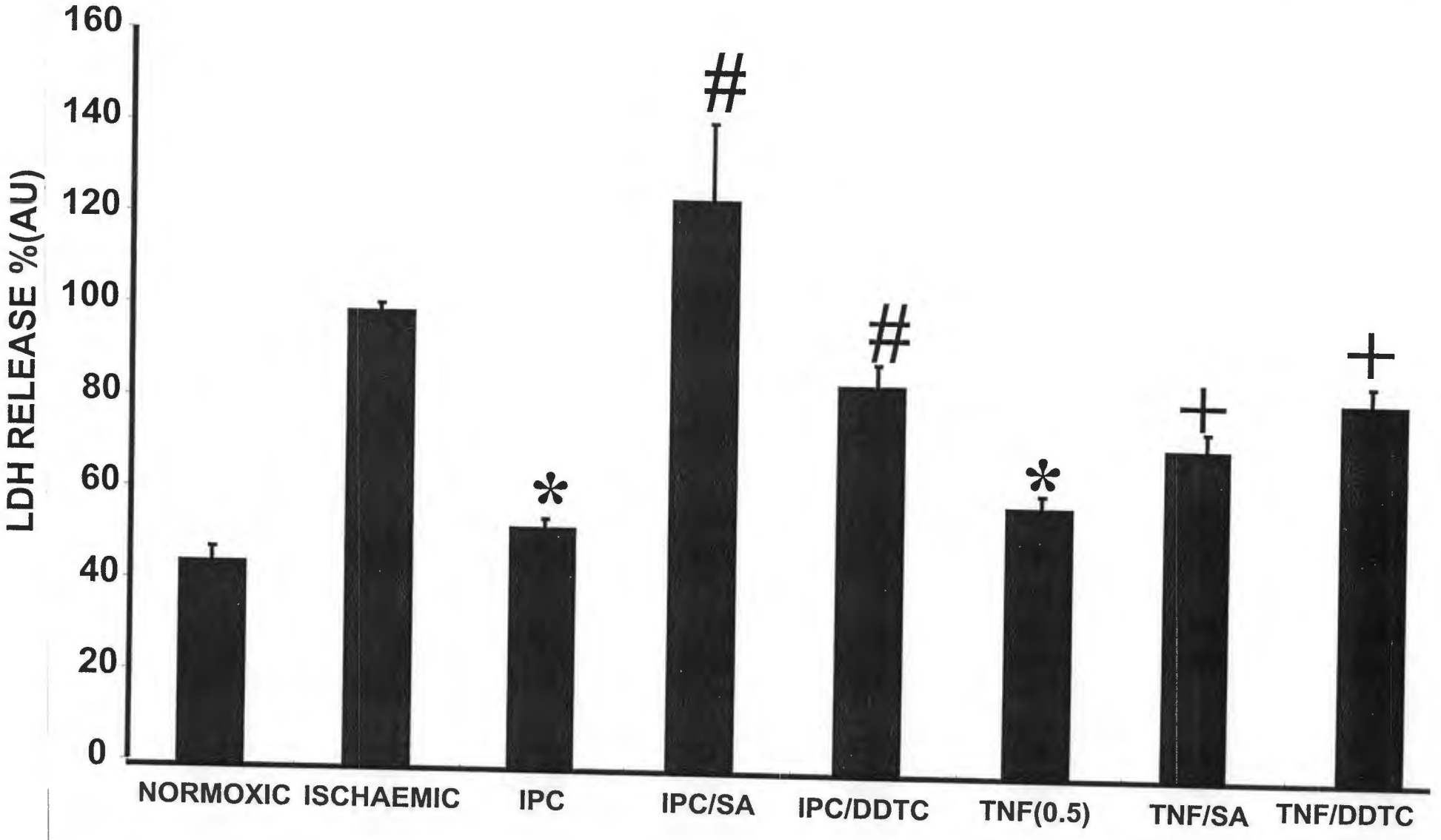
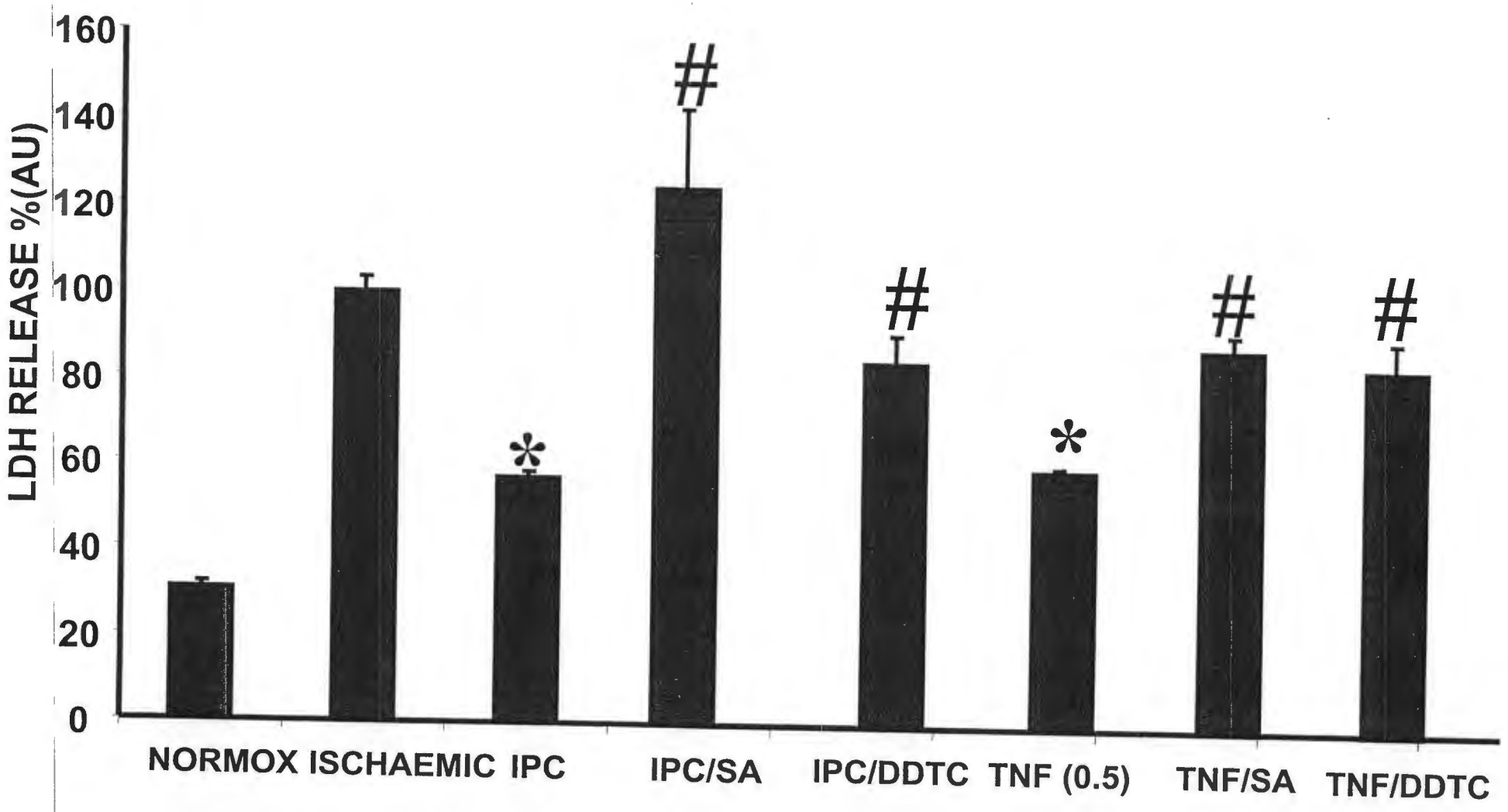


Figure 9.2

Effect of NF-κB inhibition by salicylic acid (10 mM) and diethyldithiocarbamate (DDTC; 10 mM) on ischaemic preconditioning (1 hour simulated ischaemia and 1 hour reperfusion prior to the index ischaemia) and TNFα (0.5 ng/ml) pre-treatment (30 minutes exposure followed by 1 hour wash off) on lactate dehydrogenase (LDH) release in Girardi cells following an ischaemic insult (8 hours). Data is normalised to the ischaemic insult group (100%; $n \geq 6$; *$p<0.05$ vs Ischaemic; #$p<0.05$ vs IPC and TNFα)

Fig 9.2

IPC AND TNF ARE ATTENUATED BY NFκ B ANTAGONISTS IN GIRARDI CELLS



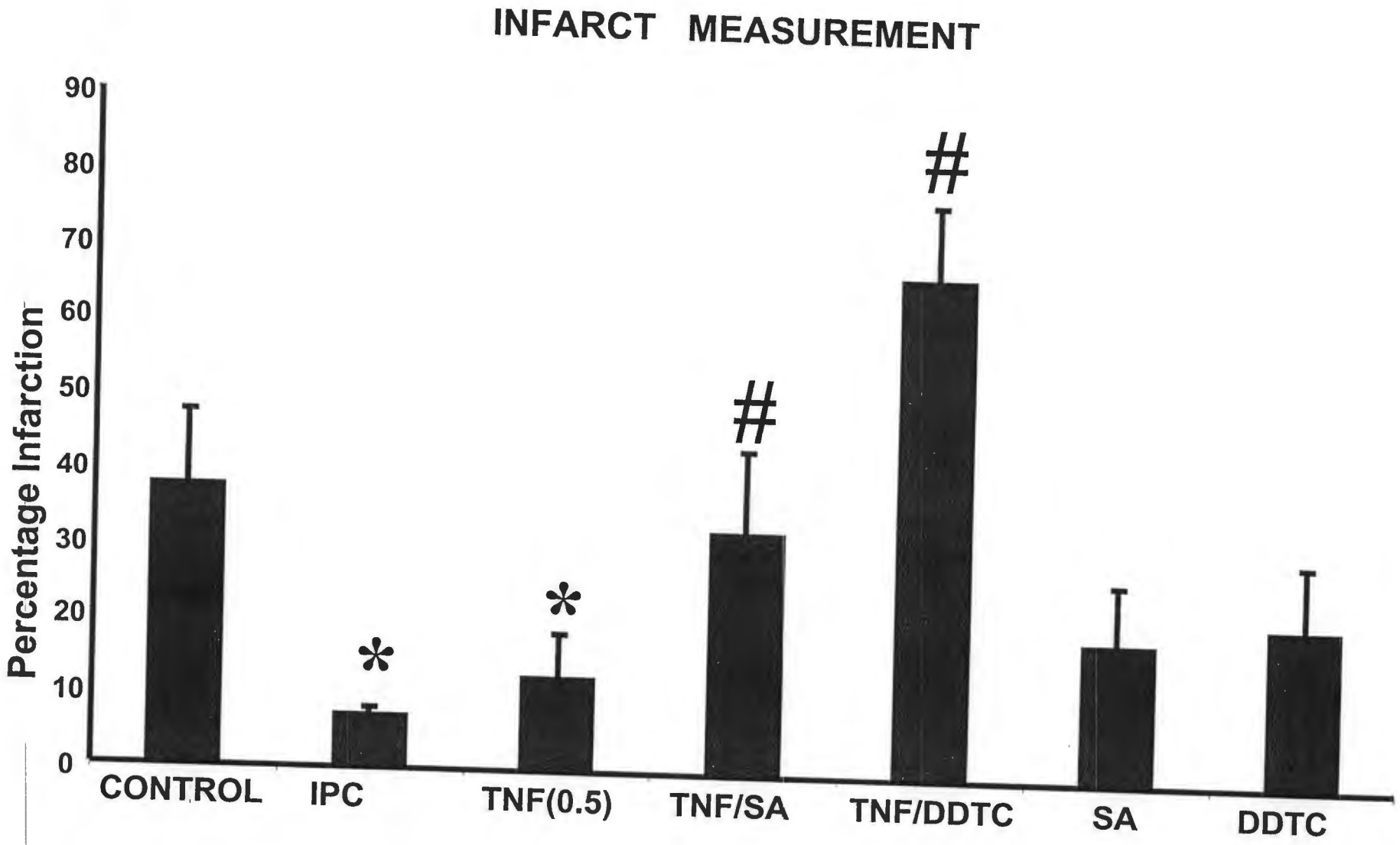
7. Infarction of IPC and TNFα versus NFκ B Antagonists

We then moved to the Langendorff perfusion apparatus to continue the study of the effect of TNFα on the rat heart. The cellular model we used identified the dose and temporal requirement of TNFα. The perfusion protocol for the isolated rat heart is illustrated in *Fig. 2*. We made use of LDH release as marker of cell damage in the cell culture model. In the rat heart perfusion model we made use of infarct size as the marker of cell damage. Our previous study suggested that IPC and TNFα might share a common mediator in the form of NFκ B in their cytoprotective effects. By shifting the system to an ex vivo model, we can clearly establish if TNFα and IPC share common signalling mediators. The TNFα PC mimetic protocol was establishes by a fellow colleague. We perfused the hearts with TNFα or with TNFα plus DDTC and SA. DDTC and SA were perfused alone to evaluate if they were toxic to the hearts. *Figure 10* demonstrates that TNFα cytoprotection can be abrogated by co-administration of the two NFκ B antagonists DDTC and SA. Our infarction data therefore support the involvement of NFκ B cytoprotective cascade of TNFα signalling.

Figure 10

Effect of NF-κB inhibition by salicylic acid (10 mM) and diethyldithiocarbamate (DDTC; 10 mM) on ischaemic preconditioning (cycles of 5 minutes global ischaemia followed by 5 minutes of reperfusion prior to the index regional ischaemia) and TNFα (0.5 ng/ml) pre-treatment (7 minutes followed by 10 minutes washout) on 30 minutes regional ischaemia (index ischaemia) followed by 2 hours reperfusion in the Langendorff perfused rat heart. Infarct size is expressed as a percentage of the area at risk. All values mean + SEM,n ≥ 6. *p<0.05 vs Control; #p<0.05 vs IPC and TNFα.

Fig 10


INFARCT MEASUREMENT
Percentage Infarction
90
80
70
60
50
40
30
20
10
0
*
*
#
#
CONTROL
IPC
TNF(0.5)
TNF/SA
TNF/DDTC
SA
DDTC

D. DISCUSSION

D. DISCUSSION

The major findings demonstrate that TNFα administration at physiological doses promotes protection against ischaemic injury in transformed cell lines and in the isolated perfused rat heart. TNFα activates this survival phenotype when administering as a PC 'trigger', as pretreatment, or if administered directly during ischaemic insult in the two cell lines studied. Finally, TNFα's protection seems to act via classical ischaemic preconditioning mediated signalling and may be activated, in part, via the transcriptional regulatory peptide NFκ B.

1. Dose and Temporal Effect of TNFα in PC in Cells

A dose response for TNFα induced preconditioning was initially determined. TNFα was shown to protect the cells against an ischaemic insult when administered at dosages ranging from 0.005 to 0.5 ng/ml. Higher doses were shown to be detrimental.[74] These data are consistent with studies in various cell types in which TNFα doses of 5.0 ng/ml up to 20.0 ng/ml have been implicated to be pro-apoptotic. Sack and colleagues have proposed the concept of physiological versus supraphysiological dose effects and state that the concentration of TNFα interacting with myocardial cells may also determine its homeostatic effect.[58] Nutt et al and Edmunds et al have illustrated the concept regarding contractile function by demonstrating that in the ex vivo heart cardiac contractile function is markedly attenuated at higher

concentrations of TNFα infusions (i.e. 20 ng/ml).[75,76] In a similar study by Yokoyama et al the concentration effect of TNFα is demonstrated in that it partially inhibited the phosphorylation of phospholamban and of troponin I.[77] We then evaluated the temporal requirement of TNFα in the two cell lines studied. The temporal effects were assessed using the optimal dose of TNF ie. 0.5 ng/ml. The TNFα concentration of 0.5 ng/ml had cytoprotective effects when administered as a PC trigger and as pretreatment in the two cell lines. Moreover, in the Girardi cells TNFα has a cytoprotective effect during the ischaemic insult. The temporal protective effect of TNFα suggests that it may act as a PC mimetic agent and that this cytokine may have anti-ischaemic properties. The latter finding is supported by the work of Mann and colleagues who demonstrated that the genetic ablation of both TNFα receptors subtypes in mice exposed to ischaemia in vivo, developed larger infarcts compared to wild-type control mice.[41]

2. Signalling with Classical IPC

Classical preconditioning results in an increased resistance to cell injury that follows short sublethal periods of ischaemia. A variety of intracellular signalling pathways have been implicated in the protective mechanism of IPC. These include the activation of G-protein-linked phospholipase C- coupled receptors, tyrosine kinase pathways, protein kinase C (PKC) and the generation of reactive oxygen species.[16] The protection confered by IPC can be blocked at several steps in each cascade. Activation of the mitochondrial

K_{ATP} channels is thought to be a pivotal intracellular event in orchestrating this protection. In our cellular model we used two K_{ATP} blockers namely glibenclamide and 5 hydroxydecanoate (5HD) to see if we can block IPC cytoprotective effect. The IPC was attenuated in both the C2C12 and the Girardi cells by glibenclamide and 5HD. Glibenclamide is however both a sarcolemmal and mitochondrial K_{ATP} channel inhibitor. Substantial evidence has accumulated in support of mitoK_{ATP} as a late effector of cardioprotection and this has shifted the focus on how the opening of a K^+ influx pathway on the inner mitochondrial membrane may be protective. Three main mechanistic hypotheses have been proposed to explain the protective effect of mitoK_{ATP} channel opening:

- Mitochondrial swelling and optimisation of Respiration
- Mitochondrial Ca^{2+} Handling and
- Free radicals and Redox state.

These are not explored in this current body of work.

3. Potential novel signalling

IPC and TNFα have similar cytoprotective effects, as is evident from their similar attenuation in LDH release in the two cell lines. Moreover, IPC and TNFα cytoprotection could be blocked by mitoK_{ATP} channel inhibitors. These observations raise the question if there is a common mediator upstream of the mitochondrial K_{ATP} for IPC and TNFα. In this regard NFκB is the first transcriptional regulator that has been identified as an integral component of the late PC.[4] NFκ B is known to be a major modulator of iNOS, COX-2 and

aldose reductase gene expression. Xuan et al has demonstrated that ischaemic PC induces a rapid activation of NFκB in rabbits. Li and colleagues have reported that NFκ B is upregulated in rat hearts subjected to short-term protocols of ischaemia/reperfusion in vivo and in vitro.[78] TNFα is known to induce NFκB activation in various tissues and cells. NFκ B activation by TNFα is via TRAF2. In this body of work we demonstrate that by using two NFκB blockers we attenuate the cytoprotective abilities of TNFα and IPC in both cell lines and in the isolated perfused rat heart. This suggests that both IPC and TNFα cytoprotection may be mediated via NFκB in our models of preconditioning.

4. Limitation of Cell Culture Model

Cell based models have certain advantages that make them useful in ischaemic reperfusion experiments. Some of the advantages are small volume of distribution, an ability to manipulate signalling proteins by introduction of cDNAs, antisense RNA, recombinant protein, interfering peptides and through the interrogation of altered signalling cascades and their consequence within a homogeneous cell type. These advantages are at the expense of a cell phenotype that differ from the intact heart and cannot be subjected to true ischaemia/reperfusion. The mechanisms may thus not reflect those in vivo. Another major differentiation factor between the cells and the in situ heart is the fact that their ischaemic periods differ from each other. Ischaemic preconditioning triggers in in-vivo and ex-vivo hearts last for about 5 minutes, but range from 30 minutes (Girardi cells) to 1 hour (C2C12

myotubes) in the cell lines. These vast differences can possibly extend what would be post-translational events in the heart into pre-translational events in the cells. Thus, it may be possible that the cytoprotection demonstrated in cells may be more relevant to delayed protection or may have a hybrid of regulatory events that span both windows of protection. In addition, cells feeding and growing result in morphological and metabolical changes. These factors might be an additional stress activated or preconditioning effect response on the cells. The C2C12 cells have a change in food substrate and it might be argued that the preconditioning effect in them might be second window related. It has previously been shown that serum deprivation can have a cytoprotective effect on the cells. In addition neither C2C12 nor the Girardi cells are true cardiac cells. The Girardi's are Hela type of cells and the C2C12 are skeletal muscle cells. This gives rise to the question as to whether the cellular mechanism of these two cell lines are relevant to the cardiac cell. The other factor that makes the two transformed cell lines different from cardiac cells is that they cannot contract. The contractility process is a very important biological process for cardiac cells and hence the morphology, enzymes and proteins differ in comparison to the transformed cells.

5. Confirmation in Perfused Hearts

To confirm TNFα's effect in the cardiac tissue, the experiments were then repeated in the isolated perfused rat heart. The isolated heart has greater physiological relevance and also for the fact that it is ex-vivo vs the in vitro transformed cell system. In the isolated rat heart other tissue come into the

picture like smooth muscle cells, myocytes, blood vessels, connective tissue and fibroblasts. All the tissues play an integral role in the heart and have the ability to release certain paracrine factors that may affect their overall biological effects. The isolated heart thus has various tissue structures compared to the transform cell lines. The main observation with the isolated heart was that the optimal TNFα (0.5 ng/ml) had the same cytoprotective abilities as it had in the cell lines. The infarction data confirms that TNFα signalling is also relevant to infarct size reduction effects of this cytokine. The dose 0.5 ng/ml confirms our hypothesis that the physiological dose is cytoprotective for both cell lines and ex-vivo contractile rat heart tissue. After it was found that TNFα could precondition the cells we investigated whether the cytoprotection might be via similar signalling paths. We used the same doses of the NFκ B inhibitors. DDTC and SA attenuated the PC mimetic cytoprotection in the isolated heart as confirmed by the increased infarct size when TNFα signalling was blocked by these agents. Thus a potential cytoprotective role might exist for TNFα in the cellular mechanism in variety of cell and tissue types.

6. Mode of Action

TNFα's mode of action that results in cytoprotection may follow a similar path as IPC. Whether this is true has to be further elucidated. As TNFα cytoprotection could be blocked by various IPC blockers, our data suggest that IPC and TNFα might have similar signalling pathways.

7. Limitation of Study Coupled to the Future Aims

The tissue culture experiments need careful attention and the cells could not be used after a few passages. With each passage the phenotypic characteristics of the cells decreased. The innate mechanism of myocardial protection has generated considerable excitement and enthusiastic research. The molecular mechanisms and the full elucidation of the signalling events are not fully understood yet. The positive side of isolated cells are the fact that they are more resilient, available and transfection permissive. The addition of mitosis and transfection with selectable markers allow the formation of stable cell lines. The draw back of the different cell lines is the inconsistent preconditioning. More and more evidence supports the contribution of K_{ATP} channels and kinase pathways in this cytoprotective process. The full detail of the process is however still to be determined and this is mostly achieved in isolated cells. Other models and tools should be developed to address certain questions and a combination of techniques are needed to confirm the mechanistic process derived from isolated cells that would also reflect the mechanistic process in vivo. The same approach that will be taken to address the complete signalling pathway of IPC could and should also be utilised to study TNFα in cytoprotection. The molecular mechanisms conferring these putative adaptive events by TNFα are still unknown and need to be investigated. Also to further explore the involvement of NFκ B in TNFα induced cardiac protection we would like to confirm the role of NFκ B using gene ablation techniques and the delineation of the NFκ B activated genes promoting cell survival. More investigation will be conducted with particular reference to the temporal activity of the cytokine in response to different

pathophysiological events and the putative divergent pathways leading either to cardiac protection or to apoptosis. The pharmacologic antagonists used in this and other studies have limitations in that they are not complete specific. Regarding this study, the molecular characterisation of the mitochondrial K_{ATP} channel is incomplete and the specificity of 5-HD is unknown. Moreover SA is known to have additional function eg. to block prostaglandin metabolism while DDTC is also known to inhibit the Cu-Zn SOD. Thus all the data derived from these pharmacologic antagonist studies need to be evaluated using different molecular and cellular approaches.

E. CONCLUSION

E. CONCLUSION

In conclusion TNFα when administered at physiological levels mimics the cytoprotective effects of ischaemic PC in cardiac and skeletal myocytes and in the isolated perfused rat heart. TNFα activates this cytoprotective programme when administered as a PC trigger as pretreatment and if administered directly during the ischaemic insult in the cell lines. These data are additional evidence to support the functional role of the innate immune system in improving cell survival in non-meyoloid tissue.

In addition, as the pharmacologic studies suggest that NFκ B signalling is important in this cell survival programme, we need to explore and characterise the downstream targets of this nuclear regulatory peptide to further advance our understanding of the innate cytoprotection. The cytoprotective properties of TNFα may be due in part to activation of the transcriptional activator NFκ B.

F. REFERENCES

F. REFERENCES

1. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation*. 1986;74:1124-1136.
2. Baxter GF, Marber MS, Patel VC, Yellon DM. Adenosine receptor involvement in a delayed phase of myocardial protection 24 hours after ischemic preconditioning. *Circulation*. 1994;90:2993-3000.
3. Bolli R. The late phase of preconditioning. *Circ Res*. 2000;87:972-983.
4. Xuan YT, Tang XL, Banerjee S, Takano H, Li RC, Han H, Qiu Y, Li JJ, Bolli R. Nuclear factor-kappaB plays an essential role in the late phase of ischemic preconditioning in conscious rabbits. *Circ Res*. 1999;84:1095-1109.
5. Cohen MV, Baines CP, Downey JM. Ischemic preconditioning: from adenosine receptor of KATP channel. *Annu Rev Physiol*. 2000;62:79-109.
6. O'Rourke B. Myocardial K(ATP) channels in preconditioning. *Circ Res*. 2000;87:845-855.
7. Fryer RM, Hsu AK, Eells JT, Nagase H, Gross GJ. Opioid-induced second window of cardioprotection: potential role of mitochondrial KATP channels. *Circ Res*. 1999;84:846-851.
8. Bell SP, Sack MN, Patel A, Opie LH, Yellon DM. Delta opioid receptor stimulation mimics ischemic preconditioning in human heart muscle. *J Am Coll Cardiol*. 2000;36:2296-2302.
9. Cohen MV, Yang XM, Liu GS, Heusch G, Downey JM. Acetylcholine, Bradykinin, Opioids, and Phenylephrine, but not Adenosine, Trigger Preconditioning by Generating Free Radicals and Opening Mitochondrial K(ATP) Channels. *Circ Res*. 2001;89:273-278.

10. Murry CE, Richard VJ, Reimer KA, Jennings RB. Ischemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischemic episode. *Circ Res*. 1990;66:913-931.

11. Fryer RM, Eells JT, Hsu AK, Henry MM, Gross GJ. Ischemic preconditioning in rats: role of mitochondrial K(ATP) channel in preservation of mitochondrial function. *Am J Physiol Heart Circ Physiol*. 2000;278:H305-312.

12. Holmuhamedov EL, Jovanovic S, Dzeja PP, Jovanovic A, Terzic A. Mitochondrial ATP-sensitive K+ channels modulate cardiac mitochondrial function. *Am J Physiol*. 1998;275:H1567-H1576.

13. Gross GJ, Fryer RM. Sarcolemmal versus mitochondrial ATP-sensitive K+ channels and myocardial preconditioning. *Circ Res*. 1999;84:973-979.

14. Tong H, Chen W, London RE, Murphy E, Steenbergen C. Preconditioning enhanced glucose uptake is mediated by p38 MAP kinase not by phosphatidylinositol 3-kinase. *J Biol Chem*. 2000;275:11981-11986.

15. Lawson CS, Downey JM. Preconditioning: state of the art myocardial protection. *Cardiovasc Res*. 1993;27:542-550.

16. Nakano A, Cohen MV, Downey JM. Ischemic preconditioning: from basic mechanisms to clinical applications. *Pharmacol Ther*. 2000;86:263-275.

17. Takano H, Bolli R, Black RG, Jr., Kodani E, Tang XL, Yang Z, Bhattacharya S, Auchampach JA. A(1) or A(3) adenosine receptors induce late preconditioning against infarction in conscious rabbits by different mechanisms. *Circ Res*. 2001;88:520-528.

18. Ping P, Zhang J, Huang S, Cao X, Tang XL, Li RC, Zheng YT, Qiu Y, Clerk A, Sugden P, Han J, Bolli R. PKC-dependent activation of p46/p54 JNKs during ischemic preconditioning in conscious rabbits. *Am J Physiol*. 1999;277:H1771-1785.

19. Ping P, Takano H, Zhang J, Tang XL, Qiu Y, Li RC, Banerjee S, Dawn B, Balafonova Z, Bolli R. Isoform-selective activation of protein kinase C by nitric oxide in the heart of conscious rabbits: a signaling mechanism for both nitric oxide- induced and ischemia-induced preconditioning. *Circ Res*. 1999;84:587-604.

20. Baines CP, Cohen MV, Downey JM. Signal transduction in ischemic preconditioning: the role of kinases and mitochondrial K(ATP) channels. *J. Cardiovasc. Electrophysiol.* 1999;10:741-754.

21. Zhao J, Renner O, Wightman L, Sugden PH, Stewart L, Miller AD, Latchman DS, Marber MS. The expression of constitutively active isotypes of protein kinase C to investigate preconditioning. *J. Biol. Chem.* 1998;273:23072-23079.

22. Ping P, Zhang J, Zheng YT, Li RC, Dawn B, Tang XL, Takano H, Balafanova Z, Bolli R. Demonstration of selective protein kinase C-dependent activation of src and lck tyrosine kinases during ischemic preconditioning in conscious rabbits. *Circ Res*. 1999;85:542-550.

23. Baines CP, Wang L, Cohen MV, Downey JM. Myocardial protection by insulin is dependent on phospatidylinositol 3-kinase but not protein kinase C or KATP channels in the isolated rabbit heart. *Basic Res Cardiol*. 1999;94:188-198.

24. Sugden PH, Clerk A. "Stress-responsive" mitogen-activated protein kinases (c-Jun N-terminal kinases and p38 mitogen-activated protein kinases) in the myocardium. *Circ Res*. 1998;83:345-352.

25. Ping P, Zhang J, Cao X, Li RC, Kong D, Tang XL, Qiu Y, Manchikalapudi S, Auchampach JA, Black RG, Bolli R. PKC-dependent activation of p44/p42 MAPKs during myocardial ischemia- reperfusion in conscious rabbits. *Am J Physiol*. 1999;276:H1468-1481.

26. Guo Y, Jones WK, Xuan YT, Tang XL, Bao W, Wu WJ, Han H, Laubach VE, Ping P, Yang Z, Qiu Y, Bolli R. The late phase of ischemic preconditioning is abrogated by targeted disruption of the inducible NO synthase gene [see comments]. *Proc Natl Acad Sci U S A.* 1999;96:11507-11512.

27. Shinmura K, Tang XL, Wang Y, Xuan YT, Liu SQ, Takano H, Bhatnagar A, Bolli R. Cyclooxygenase-2 mediates the cardioprotective effects of the late phase of ischemic preconditioning in conscious rabbits. *Proc Natl Acad Sci U S A.* 2000;97:10197-10202.

28. Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. *Annu Rev Immunol.* 1994;12:141-179.

29. Hattori Y, Nakanishi N, Kasai K. Role of nuclear factor kappa B in cytokine-induced nitric oxide and tetrahydrobiopterin synthesis in rat neonatal cardiac myocytes. *J Mol Cell Cardiol.* 1997;29:1585-1592.

30. Bolli R, Bhatti ZA, Tang XL, Qiu Y, Zhang Q, Guo Y, Jadoon AK. Evidence that late preconditioning against myocardial stunning in conscious rabbits is triggered by the generation of nitric oxide. *Circ Res.* 1997;81:42-52.

31. Sun JZ, Tang XL, Park SW, Qiu Y, Turrens JF, Bolli R. Evidence for an essential role of reactive oxygen species in the genesis of late preconditioning against myocardial stunning in conscious pigs. *J Clin Invest.* 1996;97:562-576.

32. Ping P, Zhang J, Y.T. Z, Li RCX, Guo Y, Bao W, Bolli R. Cardiac targeted transgenesis of active PKC epsilon renders the heart resistant to infarction. *Circulation.* 2000;102:II-24-25 (Abstract).

33. Imagawa J, Baxter GF, Yellon DM. Genistein, a tyrosine kinase inhibitor, blocks the "second window of protection" 48 h after ischemic preconditioning in the rabbit. *J Mol Cell Cardiol.* 1997;29:1885-1893.

34. Li RC, Ping P, Zhang J, Wead WB, Cao X, Gao J, Zheng Y, Huang S, Han J, Bolli R. PKCepsilon modulates NF-kappaB and AP-1 via mitogen-activated protein kinases in adult rabbit cardiomyocytes. *Am J Physiol Heart Circ Physiol*. 2000;279:H1679-1689.

35. Gross GJ. ATP-sensitive potassium channels and myocardial preconditioning. *Basic Res Cardiol*. 1995;90:85-88.

36. Grover GJ. Pharmacology of ATP-sensitive potassium channel (KATP) openers in models of myocardial ischemia and reperfusion. *Can J Physiol Pharmacol*. 1997;75:309-315.

37. Gross GJ, Fryer RM. Mitochondrial K(ATP) channels: triggers or distal effectors of ischemic or pharmacological preconditioning? *Circ Res*. 2000;87:431-433.

38. Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS, Heusch G, Cohen MV, Downey JM. Opening of mitochondrial K(ATP) channels triggers the preconditioned state by generating free radicals. *Circ Res*. 2000;87:460-466.

39. Forbes RA, Steenbergen C, Murphy E. Diazoxide-induced cardioprotection requires signaling through a redox-sensitive mechanism. *Circ Res*. 2001;88:802-809.

40. Downey JM, Cohen MV. Mitochondrial k(atp)channel opening during index ischemia and following myocardial reperfusion in ischemic rat hearts. *J Mol Cell Cardiol*. 2001;33:651-653.

41. Kurrelmeyer KM, Michael LH, Baumgarten G, Taffet GE, Peschon JJ, Sivasubramanian N, Entman ML, Mann DL. Endogenous tumor necrosis factor protects the adult cardiac myocyte against ischemic-induced apoptosis in a murine model of acute myocardial infarction. *Proc Natl Acad Sci U S A*. 2000;97:5456-5461.

42. Mann DL. Tumor necrosis factor and viral myocarditis: the fine line between innate and inappropriate immune responses in the heart. *Circulation*. 2001;103:626-629.

43. Medzhitov R, Janeway CA, Jr. Innate immunity: the virtues of a nonclonal system of recognition. *Cell*. 1997;91:295-298.

44. Frantz S, Kobzik L, Kim YD, Fukazawa R, Medzhitov R, Lee RT, Kelly RA. Toll4 (TLR4) expression in cardiac myocytes in normal and failing myocardium. *J Clin Invest*. 1999;104:271-280.

45. Dumitru CD, Ceci JD, Tsatsanis C, Kontoyiannis D, Stamatakis K, Lin J, Patriotis C, Jenkins NA, Copeland NG, Kollias G, Tsichlis PN. TNF-alpha Induction by LPS Is Regulated Posttranscriptionally via a Tpl2/ERK-Dependent Pathway. *Cell*. 2000;103:1071-1083.

46. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell*. 1996;86:973-983.

47. Medzhitov R, Janeway C, Jr. Innate immunity. *N Engl J Med*. 2000;343:338-344.

48. Frantz S, Kelly RA, Bourcier T. Role of TLR-2 in the activation of nuclear factor kappaB by oxidative stress in cardiac myocytes. *J Biol Chem*. 2001;276:5197-5203.

49. Belosjorow S, Schultz R, Dorge H, Schade FU, Heusch G. Endotoxin and ischemic preconditioning: TNF-α concentration and myocardial infarct development in rabbits. *Am. J. Physiol*. 1999;277:H247-H2475.

50. Nelson SK, Wong GH, McCord JM. Leukemia inhibitory factor and tumor necrosis factor induce manganese superoxide dismutase and protect rabbit hearts from reperfusion injury. *J. Mol. Cell. Cardiol*. 1995;27:223-229.

51. Greisman SE, Carozza JFA, Hills JD. Mechanisms of endotoxic tolearance. Relationship between tolerance and reticuloendothelial system phagocytic activity in the rabbit. *Exp. Med.* 1963;117:663-674.

52. Ginis I, Schweizer U, Brenner M, Liu J, Azzam N, Spatz M, Hallenbeck JM. TNF-alpha pretreatment prevents subsequent activation of cultured brain cells with TNF-alpha and hypoxia via ceramide. *Am J Physiol.* 1999;276:C1171-1183.

53. Guidot DM. Endotoxin pretreatment in vivo increases the mitochondrial respiratory capacity in rat hepatocytes. *Arch Biochem Biophys.* 1998;354:9-17.

54. Yamashita N, Hoshida S, Otsu K, Asahi M, Kuzuya T, Hori M. Exercise provides direct biphasic cardioprotection via manganese superoxide dismutase activation. *J Exp Med.* 1999;189:1699-1706.

55. Meldrum DR, Dinarello CA, Shames BD, Cleveland JC, Cain BS, Banerjee A, Meng X, Harken AH. Ischemic preconditioning decreases postischemic myocardial tumor necrosis factor-α production. Potential ultimate effector mechanisms of preconditioning. *Circulation.* 1998;98:II-214-II-219.

56. Liu J, Ginis I, Spatz M, Hallenbeck JM. Hypoxic preconditioning protects cultured neurons against hypoxic stress via TNF-alpha and ceramide. *Am J Physiol Cell Physiol.* 2000;278:C144-153.

57. Ledgerwood EC, Pober JS, Bradley JR. Recent advances in the molecular basis of TNF signal transduction. *Lab Invest.* 1999;79:1041-1050.

58. Sack MN, Smith RM, Opie LH. Tumor necrosis factor in myocardial hypertrophy and ischaemia--an anti- apoptotic perspective. *Cardiovasc Res.* 2000;45:688-695.

59. Irwin MW, Mak S, Mann DL, Qu R, Penninger JM, Yan A, Dawood F, Wen WH, Shou Z, Liu P. Tissue expression and immunolocalization of tumor necrosis factor-α in postinfarction dysfunctional myocardium. *Circulation*. 1999;99:1492-1498.

60. Meldrum DR, Cleveland JC, Cain BS, Meng X, Harken AH. Increased myocardial TNFα in a crystalloid-perfused model of cardiac ischemia-reperfusion injury. *Ann. Thorac. Surg.* 1998;65:439-443.

61. Smith RM, McCarthy J, Sack MN. TNF alpha is required for hypoxia-mediated right ventricular hypertrophy. *Mol Cell Biochem*. 2001;219:139-143.

62. Lecour SC, Minners JO, Rochette L, Opie LH, Sack MN. Tumor necrosis factor α confers cardiac preconditioning - possible role of mitochondrial K_{ATP} channel mediated event. *Circulation*. 1999;100:I-768, Abstract.

63. Liu Y, Gao WD, O'Rourke B, Marban E. Cell-type specificity of preconditioning in an in vitro model. *Basic Res Cardiol*. 1996;91:450-457.

64. Carroll R, Yellon DM. Delayed cardioprotection in a human cardiomyocyte-derived cell line: the role of adenosine, p38MAP kinase and mitochondrial K_{ATP}. *Basic Res Cardiol*. 2000;95:243-249.

65. Yaffe D, Saxel O. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature*. 1977;270:725-727.

66. Yaffe D, Saxel O. A myogenic cell line with altered serum requirements for differentiation. *Differentiation*. 1977;7:159-166.

67. Minners J, Lacerda L, McCarthy M, Meiring JJ, Yellon DM, Sack MN. Ischemic and pharmacologic preconditioning in girardi cells and C2C12 myotubes induce mitochondrial uncoupling. *Circ Research*. 2001;In Press.

68. Hearse DJ, Humphrey SM. Myocardial enzyme leakage: clearance via lymphatic and coronary routes in the isolated rat heart [proceedings]. *J Physiol.* 1979;293:4P-5P.

69. Esumi K, Nishida M, Shaw D, Smith TW, Marsh JD. NADH measurements in adult rat myocytes during simulated ischemia. *Am J Physiol.* 1991;260:H1743-1752.

70. Neely JR, Liebermeister H, Battersby EJ, Morgan HE. Effect of pressure development on oxygen consumption by isolated rat heart. *Am J Physiol.* 1967;212:804-814.

71. Awan MM, Taunyane C, Aitchison KA, Yellon DM, Opie LH. Normothermic transfer times up to 3 min will not precondition the isolated rat heart. *J Mol Cell Cardiol.* 1999;31:503-511.

72. Minners J, van den Bos EJ, Yellon DM, Schwalb H, Opie LH, Sack MN. Dinitrophenol, cyclosporin A, and trimetazidine modulate preconditioning in the isolated rat heart: support for a mitochondrial role in cardioprotection. *Cardiovasc Res.* 2000;47:68-73.

73. Yin MJ, Yamamoto Y, Gaynor RB. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. *Nature.* 1998;396:77-80.

74. Baxter GT, Kuo RC, Jupp OJ, Vandenabeele P, MacEwan DJ. Tumor necrosis factor-α mediates both apoptotic cell death and cell proliferation in a human hematopoietic cell line dependent on mitotic activity and receptor subtype expression. *J. Biol. Chem.* 1999;274:9539-9547.

75. Nutt LK, Goodwin GW, Taegtmeyer H. tumor necrosis factor-α depresses contractile function while increasing oleate oxidation in the isolated working rat heart. *Circulation.* 1998;98:I-213 (Abstract).

76. Edmunds NJ, Lal H, Woodward B. Effects of tumour necrosis factor-alpha on left ventricular function in the rat

isolated perfused heart: possible mechanisms for a decline in cardiac function. *Br J Pharmacol.* 1999;126:189-196.

77. Yokoyama T, Arai M, Sekigushi K, Tanaka T, Kanda T, Suzuki T, Nagai R. Tumor necrosis factor-α decreases phosphorylation levels of phsopholamban and troponin I in spontaneously beating rat neonatal cardiac myocytes. *J. Mol. Cell. Cardiol.* 1999;31:261-273.

78. Li C, Browder W, Kao RL. Early activation of transcription factor NF-kappaB during ischemia in perfused rat heart. *Am J Physiol.* 1999;276:H543-552.